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Pharmacokinetics in Elderly Women of Benzyl Alcohol From an Oil Depot

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ABSTRACT

Pharmaceutical oil depots are meant to release active substances at a sustained rate. Most of these depots contain benzyl alcohol (BOH) to facilitate the production and administration. Because BOH changes the solubility of components in both the body fluid and the oil formulation, it is relevant to know the change in the BOH concentration in the oil over time. In this study, volunteers were subcutaneously injected with an oil depot that contained 10% BOH, nandrolone decanoate, and cholecalciferol. The aim of this study was to determine the pharmacokinetic profiles of BOH and its metabolites benzoic acid and hippuric acid simultaneously in serum to estimate the BOH release out of the depot. For this, an HPLC bioassay was developed and adequately validated. Hereafter, the bioassay was applied to serum samples obtained at several time points between 0 and 35 days. BOH appeared immediately in serum after injection. The pharmacokinetic profile revealed that all BOH was depleted from the depot within 52 h after injection. Thus, the partition coefficient of active substances between the oil formulation and the body tissue changes rapidly in the first days after injection but will remain constant hereafter.

Introduction

Benzyl alcohol (BOH) is a commonly used excipient in oil depots in a concentration range of 1.5%-10% vol/vol.[1-4] It is used as cosolvent, local anesthetic, and as viscosity reducer in oil depots.[5] In other parenteral pharmaceutical products and in cosmetics, it is used as an antimicrobial preservative in concentrations varying from 1%-10% vol/vol.[5-7] Oil depots are meant to exhibit extended release of (active) substances.

Usually, these depots are administrated in muscular or subcutaneous tissue. Both tissues contain aqueous interstitial fluid in which the active substance and BOH are released. Although BOH is nearly always used in oil depots, there are no publications available on the release of this excipient from these formulations or on the effect of this toward the release of the drug. Recently, Kalicharan et al.[8] showed that the absorption of (active) substances from depots is determined by the partition coefficient. Because BOH changes solubility of components in both the oil and the body fluid, the distribution of (active) substances between the oil and body fluid may alter during BOH release. As a result, the absorption of these substances can be influenced during BOH release and is therefore clinically relevant.

When BOH is released from the depot, it is metabolized into benzoic acid (BA).[9] In the liver, BA is subsequently conjugated with glycine to form hippuric acid (HA).[10,11] Table 1 gives a summary of the mentioned substances.

It is important to realize that BA is also processed as such as a preservative in pharmaceutical products[5] and in food.[12,13] As there may be another source of BA than the BOH released out of the depot, it is therefore meaningful to measure the levels of BOH and its metabolites simultaneously. Until now, no analytical methods are available to separate these compounds in human serum. The

Abbreviations used: BA, benzoic acid; BOH, benzyl alcohol; CV, coefficient of variation; HA, hippuric acid; IS, internal standard.

Chemical compounds studied in this article: Benzyl alcohol (PubChem CID: 244); benzoic acid (PubChem CID: 243); hippuric acid (PubChem CID: 464); benzocaine (PubChem CID: 2337).

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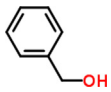
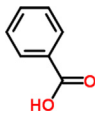
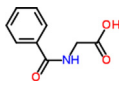
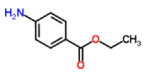
Table 1
Summary of Components Used in This Assay and Their Molecular Properties

Component Name	Molecular Formula	Molecular Structure	Molecular Weight (Da)
Benzyl alcohol	C_7H_8O		108.1
Benzoic acid	$C_7H_6O_2$		122.1
Hippuric acid	$C_9H_9NO_3$		179.2
Benzocaine (IS)	$C_9H_{11}NO_2$		165.2

Source: www.chemspider.com, visited on November 13, 2015.

HPLC method published by Tan et al.[14] to determine BOH, BA, and HA simultaneously in dog plasma was not applicable for human serum, based on our own research. This was the reason to start the development of a bioassay to determine the components of interest in human serum.

The aim of this study was to determine the pharmacokinetic profiles of BOH and its metabolites BA and HA in serum to estimate the BOH release out of the depot. This was achieved with an appropriate analytical method that enables determination of the parent and its metabolites simultaneously.

Materials and Methods

Chemicals and Reagents

BOH, BA, HA, benzocaine (internal standard, IS), and 1 M triethylammonium phosphate (pH 3.0) buffer solution were purchased from Sigma-Aldrich (St. Louis, MO). HPLC-grade methanol, acetonitrile, and perchloric acid were obtained from Merck (Darmstadt, Germany). Newborn calf serum was purchased from Life Technologies (Carlsbad, CA).

HPLC Equipment and Conditions

The separation of the analytes and IS was carried out with an isocratic HPLC method. Based on a published method by Tan et al.,[14] benzocaine (Table 1) was selected as an IS. The liquid chromatography system was from Agilent Technologies 1290 Infinity series with a diode array detector type G4212. The autosampler kept the samples at 4°C, and the injection volume was 25.0 μL. Separation was carried out on an Agilent Poroshell 120 EC-C18 (100 × 4.6-mm ID, 2.7-μm particle size) column, kept at 30°C during analysis. A phenomenex guard column (C18, 4 × 2-mm ID, 5.0-μm particle size) was used to filter out contaminants from each injection. Mobile phase composed of 40-mM triethylammonium phosphate buffer (pH 3.0), methanol, and acetonitrile (75:12.5:12.5, vol/vol/vol), and the flow rate was set at 1.0 mL/min.

A full UV spectrum analysis from 190.0 to 400.0 nm was carried out for each component (BOH, BA, HA, and benzocaine) to determine the maximal absorption (λ_{max}) and applied to the detection method (Fig. 1). Software used for equipment control and data acquisition was Chromeleon, version 7.1.3.2425 from ThermoFisher Scientific (Waltham, MA).

BOH, BA, HA, and Benzocaine Standards

A mixed stock solution of BOH, BA, and HA was prepared at a final concentration of 1 mg/mL solution in 100% methanol and further diluted in newborn calf serum to obtain working solutions of 0.1, 0.5, 1, 2.5, 5, and 10 μg/mL. IS benzocaine was freshly prepared as a 10-μg/mL solution in a mixture (1:1, vol/vol) of acetonitrile and methanol. All standards were stored at −70°C.

Sample Preparation

Two hundred-microliter serum (obtained from test subjects, see Section Test Subjects) or 200-μL working standard solutions were pipetted in 1.5-mL mixing tubes (Eppendorf, Hamburg, Germany), followed by 100-μL IS. Hereafter, 100-μL perchloric acid (10%) was added while vortexing to precipitate serum proteins. Then, samples were centrifuged for 5 min at 14,000 rpm. Supernatant was analyzed using HPLC diode array detector.

Method Validation

Identification was performed by comparing the full UV spectrum (190.0-400.0 nm) of the peaks found against a reference library (created in house). Furthermore, the software also performs a peak purity test, which gives an indication to the homogeneity of the peak. For both parameters, a match factor >85% was considered satisfactory.

Specificity was performed using blank human serum from 5 different individuals and 1 newborn calf serum sample. These samples were tested for the presence of endogenous substances coeluting with BOH, BA, HA, or IS. The observed chromatographic retention times of these 6 injections together with their full UV spectra were compared against a working standard injection to determine identity and to calculate the concentration.

Limit of quantitation (LoQ) was defined as lowest detectable concentration (0.1 μg/mL) with a signal to noise ratio of 10:1 ($n = 6$). Carryover effect was assessed by injecting a blank newborn calf serum sample after the highest quality control sample (8.0 μg/mL). Carryover in the newborn calf serum sample following the highest quality control sample should not be >20% of the lower LoQ.

Intraday accuracy and precision of the assay were evaluated together within one run (also known as repeatability[15]). Interday accuracy and precision of the assay were evaluated together on 5 different days. Newborn calf serum was spiked with BOH, BA, and HA at 4 quality control (QC) samples: 0.1, 0.5, 2.0, and 8.0 μg/mL. These concentrations were prepared in 6-fold and corresponded with LoQ, quality control low (QC_L), quality control medium (QC_M), and quality control high (QC_H), respectively. The overall bias and the coefficient of variation (CV) were calculated to evaluate the accuracy and precision of the assay.

The intraday and interday accuracy and precision for all analytes were considered acceptable for all QC samples as indicated by the overall bias (accuracy; within ±15%) and CVs (precision; <15%), respectively.[16] For LoQ, the acceptance value for the overall bias and CV was set at ±20% range and <20%, respectively.[16] A one-way ANOVA test was used for the calculations.

Recovery validation was performed using newborn calf serum and distilled water. These liquids were spiked with the mixed stock solution of BOH, BA, and HA (5 μg/mL) and IS. The recovery was calculated by dividing the peak heights of the analyte from the spiked serum by its respective counterparts from the spiked water solution (n = 4 per analyte). Recoveries of 85% or higher were considered acceptable.

Linearity validation was performed using a 6-point calibration (0.1, 0.5, 1, 2.5, 5, and 10 μg/mL) ($n = 3$ per concentration/analyte on

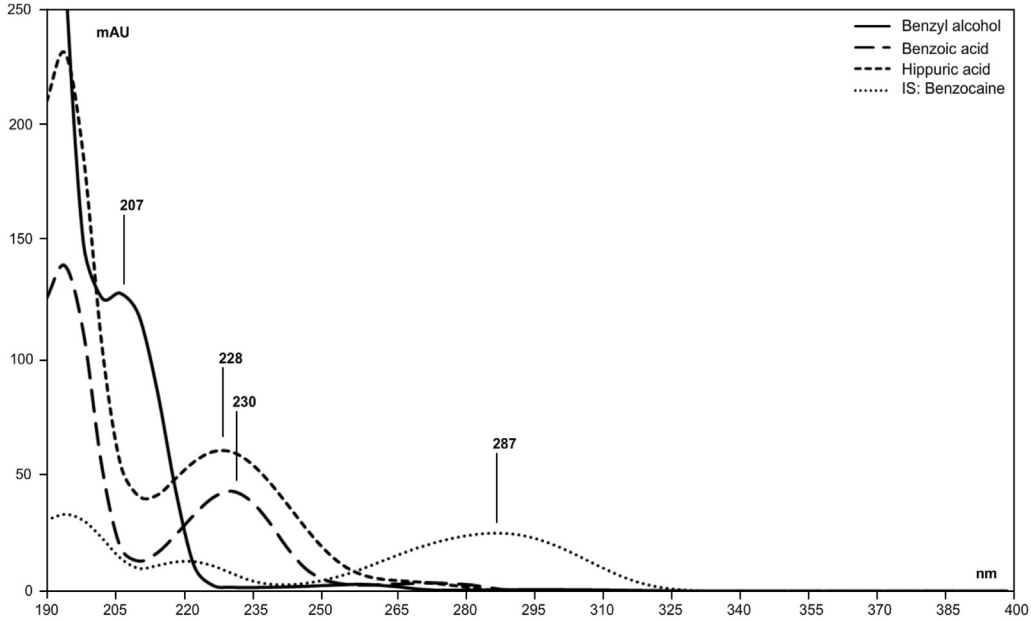


Figure 1. Full UV spectrum of benzyl alcohol, benzoic acid, hippuric acid, and internal standard (IS) benzocaine was obtained at range 190.0-400.0 nm. Characteristic peak heights for benzyl alcohol, benzoic acid, hippuric acid, and IS were 207, 230, 228, and 287 nm, respectively.

3 different days). Regression analysis of slope, intercept, and correlation coefficients (R^2) were calculated by Microsoft Excel 2010 using data analysis, regression.

Long-term stability studies of BOH, BA, and HA in newborn calf serum were evaluated at QC low and high concentrations (n = 3 per concentration/analyte). Samples were stored at −20°C for 11 months.

Freeze and thaw stability studies of the analytes were evaluated during interday accuracy tests. Freeze-thaw cycles from −70°C to ambient temperature in serum were performed 4 times, whereby samples were stored 4 h at ambient temperature. Mean concentration of each analyte at each level within ±15% of the nominal concentration was considered acceptable.

Test Subjects

Fourteen healthy female Caucasian volunteers were enrolled in Groningen (The Netherlands) in a pharmacokinetic study. The demographic characteristics were (mean ± standard deviation): age = 70.7 ± 4.3 year, length = 1.65 ± 0.1 meter, weight = 68.0 ± 9.0 kilogram, and body mass index = 24.8 ± 2.3 kg/m^2. These volunteers were initially enrolled to determine nandrolone decanoate and cholecalciferol in serum (see below for more information about the oil depot formulation and study). Residual serum samples from the same volunteers were used in the present study.

There was no loss to follow-up, and no adverse reactions were reported during this study. Ethics approval for the study was obtained from the Central Committee on Research Involving Human Subjects in the Netherlands. All volunteers signed the informed consent to participate in the study. Serum samples were collected at QPS Netherlands B.V. (Groningen, The Netherlands). All volunteers received a 0.5-mL oil depot, subcutaneously injected in the upper arm. This oil depot was manufactured under current good manufacturing practice conditions in the hospital pharmacy at the University Medical Center Utrecht, The Netherlands. It contained 0.93 mmol/mL (=100 mg/mL) BOH, 50 mg/mL nandrolone decanoate, and 28,000 IU/mL cholecalciferol in sesame oil. The sustained release of nandrolone decanoate and cholecalciferol were studied in a phase 1 clinical trial. The influence of BOH on the absorption of nandrolone is published elsewhere.[8]

Samples were taken directly after injection (0 h) and at 2, 4, 8, 12, 15, 22, 24, 36, 48, 72, 96, 168, 216, 264, 360, 456, 552, 648, and 840 h after injection. Samples were stored at −70°C until they were analyzed for BOH, BA, and HA at the Clinical Pharmacy, University Medical Center Utrecht, the Netherlands.

Data Analysis of Pharmacokinetic Study

Data will be depicted as concentration-time curves (mean ± SEM). Area under the curve (AUC) was calculated using the trapezoidal rule. The maximum serum concentration (C_{max}) and the time to reach maximum serum concentration (T_{max}) were obtained directly from the concentration-time curve.

Results and Discussion

This study was initiated to develop and validate a bioassay for BOH, BA, and HA in human serum by HPLC. Hereafter, this assay was applied to serum samples from healthy volunteers to study the pharmacokinetic profiles of BOH and its metabolites.

Analytical Method Development

A previously published liquid chromatography-tandem mass spectrometry method by Penner et al.[17] for BA and HA analysis in urine was examined. This method, using an alkaline buffer, provides stable ions for BA and HA. It was checked whether this method is applicable for BOH analysis. Unfortunately, neither positive nor negative BOH ions were formed with an acidic or with an alkaline mobile phase. In addition, formation of positive or negative adducts of BOH with ammonium formate or LiCl did not result in a successful analytical method either.

Three other published gas chromatography methods[18-20] turned out to be inadequate as well: without derivatization, BA appeared to be difficult to separate due to excessive peak tailing. Finally, several HPLC methods[14,21,22] were published for BOH, but unfortunately none were applicable for serum analysis of all components

simultaneously. Tan et al.[14] published an HPLC method to determine all three components in dog plasma. Unfortunately, our own research showed an undesirable resolution of the 3 components spiked in calf and human serum. HPLC methods published by Sudareva et al.[21] and Pérez-Lozano et al.[22] did not include HA, which was required to meet our aim.

The here-developed method exhibits the benefit of having a fast and simple sample cleanup, which was able to measure the levels of BOH and its metabolites in human serum simultaneously.

Method Validation

The developed analytical method was validated by evaluating specificity, identification, accuracy, precision, LoQ, recovery, carryover effect, linearity, and stability. This assay was validated in accordance to the latest criteria found in the ICH Q2(R1) guideline, the EMA Guideline for bioanalytical method validations and literature.[15,16,23] All studied validation parameters met the predefined acceptance criteria.

The 5 blank human serum samples and the newborn calf serum samples were tested for specificity. BOH concentrations in all blank serum samples were below LoQ (see Supplementary Data). All blank samples contained HA and BA. HA and BA concentrations (mean ± SEM) found in the blank human samples were 0.8 (± 0.5) and 0.05 (± 0.01) μg/mL, respectively. Newborn calf serum contained 0.24 (± 0.01) and 0.06 (± 0.01) μg/mL HA and BA, respectively. Therefore, newborn calf serum was preferred as the matrix for our standard solutions because of the lower amounts of HA.

No interference peaks for BOH and BA were found in the chromatogram originating from the calf serum used (Fig. 2 and Supplementary Data). The analysis was adequate for all HA concentrations, except for the LoQ concentration (=0.1 μg/mL) in the calibration curve (see Supplementary Data). However, all HA concentrations in the conducted clinical study were accepted because they were all above 0.5 μg/mL. Spectral match and peak purity data from the samples used in the pharmacokinetic study were above 85% for all analytes (Table 2). Peak purity of BOH at LoQ concentration (=0.1 μg/mL) was 71.0%, however. As can be seen in the clinical study in the following section, all BOH concentrations were above 0.5 μg/mL. Therefore, this peak purity was considered as clinically irrelevant for this study. IS peak purity for the nominal concentrations 0.1, 0.5, 2.0, and 8.0 μg/mL showed purities of 97.7%, 97.3%, 96.9%, and 97.3%, respectively.

The results of the accuracy and precision for the 3 components are summarized in Table 2. The intraday and interday accuracy (overall bias) were determined by repeated analysis of 6 spiked newborn calf serum samples. The replicated analyses varied between −2.4% and +9.1%. The intraday, interday, and overall precision (% CV) were <9.8% for the QC samples and <18.4% for the LoQ spiked samples.

LoQs of all analytes in this assay were 0.1 μg/mL, which equals molar concentrations of 0.93, 0.82, and 0.56 nmol/mL for BOH, BA, and HA, respectively. LoQ for the developed bioassay was comparable or superior to other methods.[17,24] No carryover effect in newborn calf serum from QC high was measured.

The recoveries of BOH, BA, HA, and IS were (mean ± relative standard deviation) 96.3% (± 0.7), 87.5% (± 0.6), 103% (± 0.7), and 98% (± 0.7) respectively, which is in line with literature data.[14]

The linearity for the 3 analytes was evaluated over the range 0.1-10 μg/mL. Regression coefficients were all ≥0.999.

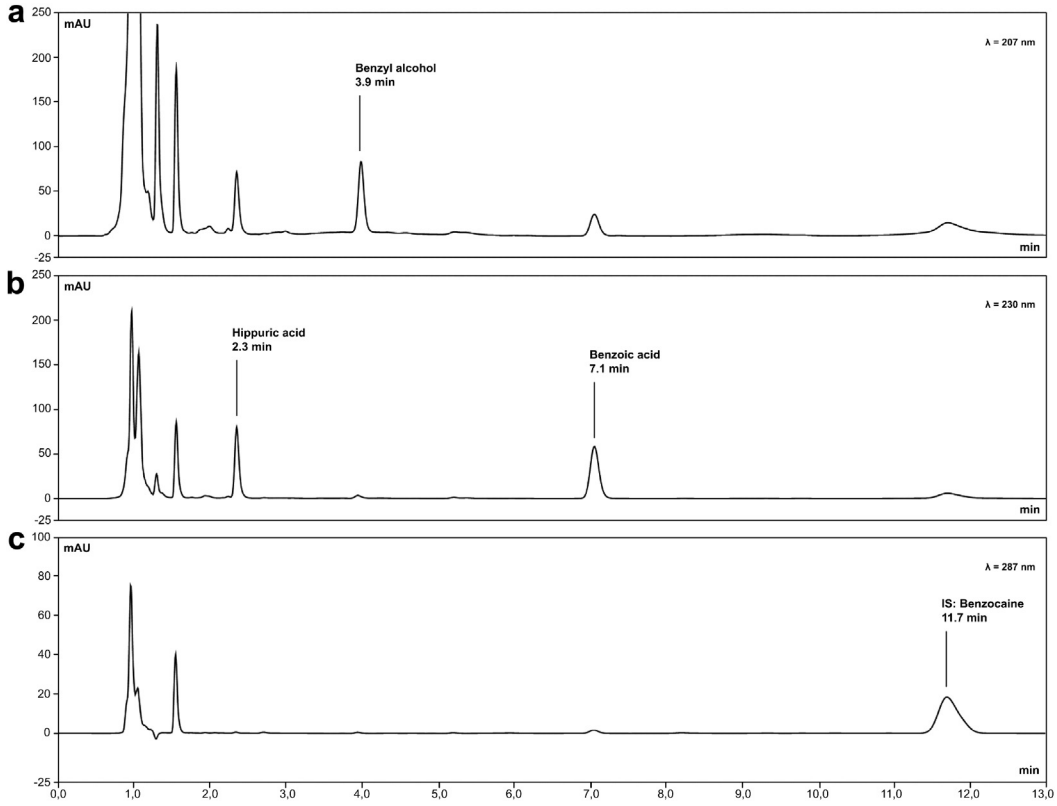


Figure 2. Three representative HPLC chromatograms obtained from one analytical run. Analyzed sample contained newborn calf serum spiked with the 4 studied components (8.0 μg/mL): (a) benzyl alcohol at wavelength (λ) 207 nm; (b) benzoic acid and hippuric acid at λ = 230 nm; and (c) benzocaine was measured at 287 nm. All components were well separated from endogenous substances and from each other.

Table 2
Overview of Intraday and Interday Precision, Accuracy, and Stability of Benzyl Alcohol, Benzoic Acid, and Hippuric Acid

Compound	Nominal Concentration (μg/mL)	Peak Purity (%)	Intraday (n = 6) CV (%)	Interday (n = 5) CV (%)	Overall CV (%)	Overall Bias (%)	Stability (%)
BOH	LoQ (0.1)	71.0	2.3	18.3	18.4	9.1	–
	QC_L (0.5)	98.1	1.9	3.5	4.0	−2.4	−8.6
	QC_M (2.0)	99.9	1.1	0.8	1.4	3.2	–
	QC_H (8.0)	100.0	0.5	2.2	2.2	−0.3	−1.0
BA	LoQ (0.1)	99.7	17.7	0.0	17.7	3.6	–
	QC_L (0.5)	100.0	7.6	0.0	7.6	3.3	−3.9
	QC_M (2.0)	100.0	0.7	1.2	1.4	4.2	–
	QC_H (8.0)	100.0	0.6	1.5	1.6	−0.8	−1.3
HA	LoQ (0.1)	99.3	1.6	5.0	5.2	8.9	–
	QC_L (0.5)	99.8	1.1	2.0	2.3	0.9	2.5
	QC_M (2.0)	100.0	9.8	0.0	9.8	5.9	–
	QC_H (8.0)	100.0	1.1	1.7	2.1	−1.1	−2.7

All long-term stability (11 months at −20°C) results were <± −8.6% (Table 2). Freeze-thaw stability was performed during interday accuracy and varied between −2.4% and +9.1% (Table 2).

Pharmacokinetic Profiles of BOH, BA, and HA in Serum

The here-developed HPLC method was applied to analyze BOH, BA, and HA in human serum samples. Figure 3 shows the mean serum concentration-time profiles of BOH and its metabolites for the entire study period. The pharmacokinetic parameters are summarized in Table 3.

BOH levels appear directly in serum after subcutaneous injection, which shows that BOH is released immediately from the oil depot (Fig. 3a). In general, the drug serum level is a net result of drug released from the oil depot and the drug elimination out of the central compartment. Here, the BOH serum concentrations decreased during the first 52 h after injection, except for the period of 12-24 h after injection (Fig. 3b). In this period, a remarkable increase in BOH concentration was observed. To explain this phenomenon, one can speculate that the depot might exhibit a temporary higher release or that there is a temporary change in clearance. It should be noted that all volunteers received the injection between 9 and 11 o'clock in the morning. It is likely that the volunteers slept in the period 12-24 h after injection. It can be speculated that the mass transfer of the component out of the oil depot changes because of a certain body position, local pressure, or even an increased body temperature. The latter, however, is not very likely; Gillberg and Akerstedt[25] reported that body temperature does not increase during sleep, thus this parameter would not affect the mass flux of BOH in this study. A change in the elimination may occur, but there is no evidence for a changed enzyme activity of BOH oxidation during the night. Finally, enterohepatic circulation of BOH can be excluded because its molecular weight is much lower than the threshold value of 500 for the biliary route of excretion.[26]

Figure 3 shows that serum levels of BA increase when serum levels of BOH decline. This is what can be expected from the metabolism of BOH. After conjugation with glycine, BA forms HA.[27] HA is excreted from the body via the renal route.[27,28] Although the reaction rates are unknown, it is possible that the conversion of BA to HA occurs faster than the oxidation process of BOH to BA. This could explain the more than 10-fold molar concentration differences between BOH and BA. $AUCs_{0\text{-}840h}$ of BOH were similar to those of BA with respective values of 397.3 ± 44.9 nmol/mL × h and 417.4 ± 63.2 nmol/mL × h. This indicates that all BOH was metabolized to BA and that apparently there is no other source of BA than the BOH.

The serum level of HA was 5.8 nmol/mL immediately after injection (Fig. 3). As described in the previous section, HA is formed in the liver by conjugation of BA with a glycine molecule. Yet, no BA

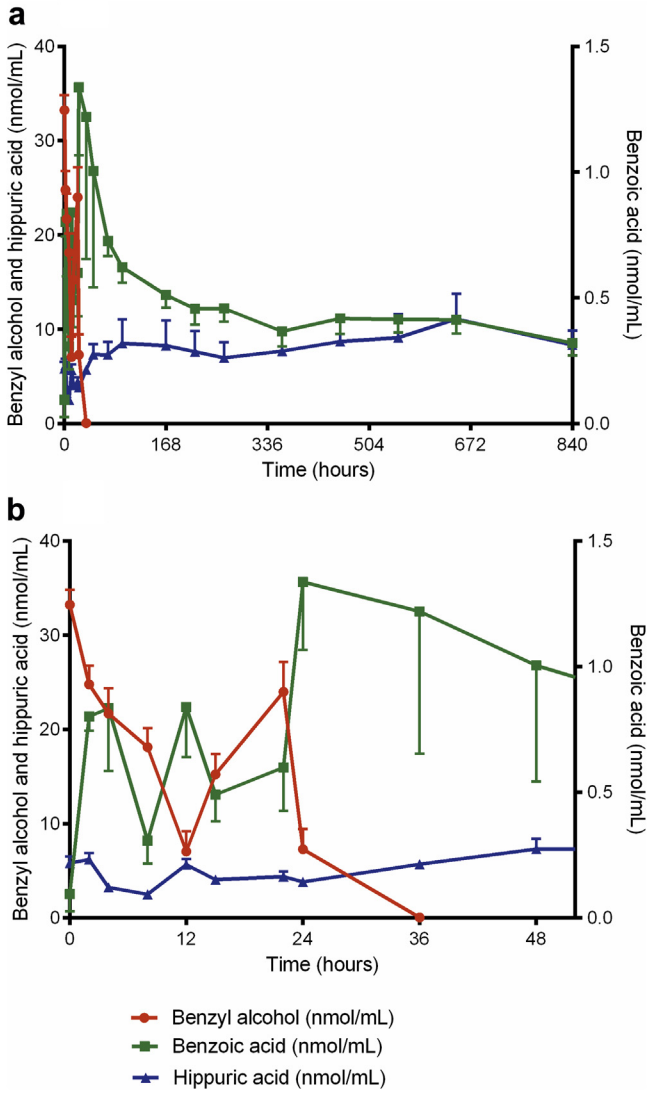


Figure 3. Pharmacokinetic profiles of benzyl alcohol (red line), benzoic acid (green line), and hippuric acid (blue line) in serum after injection, presented as mean ± SEM. Healthy females (n = 14) received subcutaneously 50-mg benzyl alcohol dissolved in an oil depot. Notice that the left *y*-axis shows the values for benzyl alcohol and hippuric acid and the right *y*-axis presents the values for benzoic acid. Upper figure (a) presents the entire study period (840 h), whereas the first 52 hours after injection are illustrated at the bottom of this figure (b).

Table 3
Pharmacokinetic Parameters (Mean ± SEM) of BOH, BA, and HA After Subcutaneous Administration of 0.46 mmol BOH in Human (n = 14)

Substance	$AUC_{0\text{-}840h}$ (nmol/mL × h)	C_{max} (nmol/mL)	T_{max} (h)
BOH	397.3 ± 44.9	34.8 ± 1.5	3.3 ± 2.1
BA	417.4 ± 63.2	1.8 ± 0.4	36.3 ± 18.0
HA	7113.6 ± 1435.9	16.6 ± 2.7	374.6 ± 86.6

was determined at t = 0, implying this reaction has not taken place. Therefore, it is obvious that HA is present in the body as a metabolite of other (body/food) substances, as has been argued earlier.[29-31] In contrast to BA, there is another source for HA other than BOH.

The major goal of the present study was to determine the pharmacokinetics of BOH from the oil depot. As shown in Figure 3, this excipient can only be measured in serum during only a few days. This means that the formulation that contains 10% of BOH at the moment of injection changes dramatically within this period. BOH acts as a cosolvent in oil, which means that the solubility of ingredients is positively affected by its presence. When the BOH is fully depleted, the partition coefficient of these ingredients between the oil formulation and the body fluids will be changed, which means that the driving force for diffusion (i.e., the rate of release) has altered. Whether this is directly related with a change in drug absorbance (i.e., the appearance in the central circulation) is not fully clear at this point because there are more variables of relevance in determining the drug absorption rate from the depot. The transfer through the body tissue may also have a significant influence on the absorbance of the active substances. For this reason, it is of interest to monitor the absorption of active ingredients in the very first days of *in vivo* release. This is reported in a separate article.[8]

Influence of Other Factors on Serum Levels on Components of Interest

As mentioned previously, BOH and BA are processed as a preservative in several pharmaceutical drug products. BOH is a naturally occurring compound in food, for example, in some fruits (<5 mg/kg), black tea (1-15 mg/kg), and green tea (1-30 mg/kg).[32] As reported by the Scientific Commission of the European Union, the maximally allowed quantity of BOH in end products (foods and beverages) is 300 mg/kg.[32] Although the quantities that can be consumed can be relatively high, these were apparently not taken by the volunteers as levels of BOH were below LoQ before depot injection as well as 52 h after injection.

Serum levels of BA and HA may emerge from other sources. However, because BOH and BA levels correspond with each other and their AUCs are comparable, it is likely that the measured BA is mainly originated from BOH released from the depot.

Conclusions

This article reports the development of an assay for simultaneous determination of BOH, BA, and HA in human serum by HPLC. The assay is tested and validated to conform to the latest ICH Q2(R1) guideline and EMA Guideline for bioanalytical method validations. It appears to be accurate, selective, sensitive, and reproducible for BOH, BA, and HA in human serum. The sample pretreatment is simple to perform because no preconcentration, derivatization, or extraction procedures are required. In addition, it has a lower LoQ compared to published methods using LC-MS/MS.

The newly developed method was applied to determine the pharmacokinetics of BOH released from a subcutaneously injected oil depot, which contained 10% BOH, nandrolone decanoate, and cholecalciferol. The pharmacokinetic profile revealed that all BOH was released from the depot within 52 h after injection. This means that the release of the (active) substances from the oil depot may be affected in this period and therefore also the absorption *in vivo*. Interestingly, 2 peak levels of BOH were determined in all volunteers. Further studies are needed to determine the cause of this phenomenon. A raise in BA level is observed simultaneously with the decrease in BOH level, which can be explained by the mechanism of metabolism.

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