Specimen handling in an HPLC determination of phenylbutazone and its major metabolites in plasma, avoiding degradation of the compounds

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Introduction
Since its introduction in 1949 phenylbutazone has been widely used as an anti-inflammatory drug and has proved to be an effective analgesic. Recently because of hematologic side effects of phenylbutazone its use has been restricted to ankylosing spondylitis.1 Soon after its introduction Burns et al. described a spectrophotometric method for the determination of phenylbutazone in biological fluids.2 They also demonstrated oxidative metabolic pathways yielding oxyphenbutazone and gammahydroxyphenylbutazone.2 Since then several other methods for the determination of phenylbutazone and its metabolites have been described, including spectrophotometric methods,2 gas liquid chromatographic methods5-11 and high pressure liquid chromatographic methods.12-16 Each of these methods has its disadvantages. Some of these methods are only suitable for the determination of phenylbutazone. In general the problems are lack of sensitivity or selectivity and especially the fact, usually overlooked, that phenylbutazone and its metabolites can degrade to a substantial degree.17-20 This degradation occurs not only upon storage, but also on extraction under acidic conditions, especially when the temperature is raised. Therefore the handling of the biological samples during the extraction procedure is critical.

The essential point of our newly developed method for measuring phenylbutazone and its oxidative metabolites is that extreme care has been taken to avoid degradation during sample preparation. In this procedure 0.345 M citrate buffer (pH 2.0) is used to acidify the plasma sample, instead of the common 1-5 N HCl, and the extraction tubes are centrifuged at 4°C. Feprazone is used as an internal standard. The method was successfully applied in human studies.

Methods

REAGENTS AND DRUGS

Methanol, citric acid, sodium hydroxide, hydrochloric acid, diethyl ether, n-hexane, sodium hydrogen carbonate, tris(hydroxymethyl)aminomethane and i-butanol were obtained from Merck (Darmstadt, FRG). Diethyl ether was distilled shortly before use. Tetrabutylammonium hydrogen sulfate was obtained from Janssen Chimica (Beerse, Belgium). All reagents were of analytical grade quality. Commerciably available dosage forms of phenylbutazone (Butazolidin Alka®) and oxyphenbutazone (Tanderil®) were used. Gammahydroxyphenylbutazone and feprazone were kindly supplied by Ciba-Geigy (Arnhem, The Netherlands) and C.H. Boehringer Sohn (Ingelheim am Rhein, FRG), respectively.

BIOLOGICAL FLUIDS

Human blood plasma stored at -20°C was obtained from the local blood bank. From one male individual blood was collected in heparinized containers and centrifuged to separate the plasma after a single oral dose administration of two 100 mg Butazolidin Alka® tablets. From another male individual plasma was obtained in the same manner.
after a single oral dose administration of two 300 mg Tanderil® tablets. Furthermore, plasma samples were collected from eleven patients with ankylosing spondylitis during a trial comparing diflunisal with phenylbutazone. All patients used phenylbutazone for at least three months.

A verbal informed consent was obtained from all patients and volunteers. The study was approved by the Ethics Committee of the Medical Faculty of the University of Nijmegen.

**APPARATUS AND CHROMATOGRAPHIC CONDITIONS**

A Hewlett-Packard (Hewlett-Packard, Waldbronn, FRG) HP 1084 B liquid chromatograph equipped with an automatic sampling system was used. The compounds eluted were measured with a variable wavelength detector (Spectroflow 773, Kratos Analytical Instruments, New Jersey, USA). The detection wavelength was 248 nm. The stainless steel column (15 cm × 4.6 mm i.d.) was packed with LiChrosorb RP18, mean particle size 5 µm (Merck). The oven temperature was 35°C and the injection volume was 10 µl.

The mobile phase was a mixture of methanol, 1-butanol and bidistilled water (37:6+6:57) containing 0.0102 M tetrabutylammonium hydrogen sulfate and 0.0098 M tris(hydroxymethyl)aminomethane (pH 4.0). This was delivered at a rate of 1.0 ml/min, producing a pressure of 21.8 MPa. The mobile phase was prepared as follows: 3.74 g of tetrabutylammonium hydrogen sulfate (mol.wt. = 339.54) were dissolved in 314 ml bidistilled water and 1.33 g tris(hydroxymethyl)aminomethane (mol.wt. = 121.14) were dissolved in 314 ml bidistilled water. The latter solution was added to the tetrabutylammonium hydrogen sulfate solution until pH 4.0 was attained (303 ml were required). Next, the mixture was filtered through a 0.45 µm Millipore filter, and 370 ml methanol and 60 ml 1-butanol were subsequently added to 570 ml of the filtered mixture.

**PROCEDURE**

Into an extraction tube, which already contained 200 µl methanol (also containing phenylbutazone, oxyphenbutazone and gammahydroxyphenylbutazone when making calibration curves) 100 µl of internal standard (10 or 50 µg per 100 ml methanol in single dose or steady-state studies, respectively) were pipetted. Aliquots of 0.5 ml of plasma, 0.7 ml of 0.345 M citrate buffer pH 2.0 and 5 ml of diethyl ether–n-hexane (50:50) were successively pipetted into the extraction tube.

The extraction tube was closed with a polytetrafluoro-ethylene (PTFE) screw-cap. After whirlmixing for 2 s the tubes were tumbled mechanically for 30 min at 12 rpm at room temperature, followed by centrifugation at 13000 g for 30 min at 4°C (Minifuge 2, Heraeus-Christ GmbH, Osterode am Harz, FRG). The organic layer was pipetted into another extraction tube containing 100 mg sodium hydrogen carbonate. The tube was closed with a screw-cap and tumbled by hand for 12 s. The screw-cap was opened to let the CO₂ formed escape (12-15 min), followed by a gentle whirlmixing for 2 s. After centrifugation at 1300 g for 5 min at 4°C, the organic layer was transferred to a test tube and evaporated to dryness with a gentle stream of nitrogen at 25°C. The residue was dissolved in 1.0 ml of methanol. After addition of 0.2 ml of bidistilled water, 10 µl of this mixture were injected into the column.

**RESULTS**

Figure 1 shows typical chromatograms for blank plasma and for plasma containing 10.0 µg/ml gammahydroxyphenylbutazone, 10.0 µg/ml oxyphenbutazone, 20.0 µg/ml phenylbutazone and 20.0 µg/ml of the internal standard feprazone. The retention times of gammahydroxyphenylbutazone, oxyphenbutazone, phenylbutazone and feprazone were about 5.72, 6.86, 16.94 and 20.10 min, respectively. Most of the endogenous peaks had a retention time shorter than 4 min, one endogenous peak between the peaks of oxyphenbutazone and phenylbutazone had a retention time of 11.0 min.

Figure 2 shows chromatograms for plasma containing 20 µg/ml phenylbutazone and 10 µg/ml oxyphenbutazone using 0.5 ml 1 N HCl instead of 0.7 ml 0.345 M citrate buffer pH 2.0. A considerable degradation occurred during the extraction procedure resulting in decomposition products with a retention time of 13.91 min for phenylbutazone, and