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Solid-phase extraction for the high-performance liquid chromatographic determination of indomethacin, suxibuzone, phenylbutazone and oxyphenbutazone in plasma, avoiding degradation of compounds

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ABSTRACT

A solid-phase extraction method was validated for the simultaneous high-performance liquid chromatographic determination of indomethacin, suxibuzone, phenylbutazone, oxyphenbutazone and their degradation products. Indomethacin was added as internal standard to plasma samples, which were then acidified with citrate buffer and passed through a phenyl cartridge. The drugs were eluted with hexane–diethyl ether (1:1, v/v), and the organic extract was taken to dryness. The residue was dissolved in methanol and chromatographed on a C_{18} column with ultraviolet detection at 240 nm. The elution was isocratic with a mobile phase of 0.02 *M* ammonium sulphate–acetonitrile (45:55, v/v), pH 3. For indomethacin, suxibuzone, phenylbutazone and oxyphenbutazone the limit of quantitation was 0.05 μg/ml. The linearity was checked between 0.05 and 100 μg/ml ($r = 0.999$); within this range the recovery was higher than 90% and the accuracy showed relative errors of less than 7.5%. The main advantage of this method is the avoidance of degradation by using citrate buffer instead of the usual 5 *M* hydrochloric acid to acidify the plasma. The method is also more specific and less time-consuming than the previously reported liquid–liquid extraction, and could possibly be automated.

INTRODUCTION

Suxibuzone [4-butyl-4-(hydroxymethyl)-1,2-diphenyl-3-pyrazolidinedione hydrogen succinate] is an anti-inflammatory agent, derived from phenylbutazone. Suxibuzone is metabolized in the body mainly to phenylbutazone, and then to oxyphenbutazone and hydroxyphenylbutazone.

A linear gradient high-performance liquid chromatographic (HPLC) assay for suxibuzone and its metabolites in plasma and urine has been reported [1]. In this method a double extraction with benzene–cyclohexane (1:1, v/v) and 5 *M* hydrochloric acid to acidify the samples (to pH 2) was used.

Many methods have been proposed for the quantitative assay of phenylbutazone and its metabolites in biological fluids by gas chromatography (GC) [2–6] and HPLC [7–12]. The main disadvantage of most of these methods is the degradation of phenylbutazone and its metabolites, not only during storage but also when they are extracted in acidic conditions [12–15]. In a recent paper, a quite complex solid–liquid extraction was described for the determination of phenylbutazone only [16].

This paper describes a simple HPLC method

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using solid-phase extraction, to avoid degradation during the sample preparation, for the simultaneous determination of suxibuzone and its metabolites. Indomethacin was used as the internal standard. The method was successfully applied in human studies.

EXPERIMENTAL

Chemicals and reagents

Suxibuzone was supplied by Laboratorios Dr. Esteve (Barcelona, Spain) and phenylbutazone, oxyphenbutazone and indomethacin were all obtained from Sigma (St. Louis, MO, USA). Diethyl ether, 2-propanol, ammonium sulphate, sulphuric acid, citric acid and sodium citrate were of analytical-reagent grade (Merck, Darmstadt, Germany). Benzene was of analytical grade (Fluka, Buchs, Switzerland), as were cyclohexane and hydrochloric acid (Normasolv, Barcelona, Spain). Hexane, methanol, acetonitrile, ethyl acetate and chloroform were of HPLC grade (Scharlau, Barcelona, Spain). The organic solvent used for extraction was prepared by mixing hexane and diethyl ether (1:1, v/v). Bond Elut PH columns were provided by Analytichem International (Harbor City, CA, USA).

Experimental standard solutions

Stock standard solutions of suxibuzone, phenylbutazone, oxyphenbutazone and indomethacin were prepared in methanol at a concentration of 1 mg/ml and were stored at −20°C. Secondary standard solutions from the four compounds were obtained by diluting 1:100 with methanol at a concentration of 10 μg/ml. These solutions were prepared weekly, checked by UV spectrophotometry every day, and stored at 4°C.

Indomethacin and suxibuzone solutions are more stable, whilst phenylbutazone and oxyphenbutazone are easily degradable in solution.

Chromatographic system

Apparatus. The HPLC analyses were performed using a modular liquid chromatographie system consisting of a Spectroflow 400 pump and a Spectroflow 783 UV detector from Applied Biosystems (Foster City, CA, USA), together with a Promis II autosampler (Spark-Holland, Emmen, Netherlands). Data collection and reduction were performed using a computing integrator program (Nelson Analytical 2600 Version 5, Perkin Elmer Nelson System).

Column and mobile phase. Separations were performed on a C_{18} reversed-phase column (Ultrasphere ODS, 5 μm, 15 cm × 0.46 cm I.D., Beckman). A C_{18} pre-column (Nova-Pack, 4 μm, 6 mm × 4 mm I.D., Waters) was also used. The mobile phase was acetonitrile–0.02 *M* ammonium sulphate (55:45, v/v). The chromatographic separation was performed isocratically at ambient temperature at a flow-rate of 1.5 ml/min, with UV detection at 340 nm.

Extraction procedure for plasma samples

Phenyl cartridges were conditioned with 5 ml of methanol and 5 ml of water. Plasma samples of 500 μl were spiked with 50 μl of 40 μg/ml internal standard solution (2 μg of indomethacin), then adjusted to pH 3.4 with 0.345 *M* citrate buffer and passed through a phenyl sorbent. The cartridge was washed with water, dried, and eluted with 5 ml of hexane–diethyl ether (1:1, v/v). The organic phase was brought to dryness under a stream of nitrogen at 40°C. The dry residue was redissolved with 200 μl of methanol, and 25 μl were injected into the chromatograph.

RESULTS

Representative chromatograms of suxibuzone (S), phenylbutazone (P), oxyphenbutazone (O) and indomethacin (I) (internal standard) are given in Fig. 1. Their respective retention times were 6.2, 9.8, 4.0 and 7.5 min.

Linearity

The linearity of the method was tested in order to demonstrate a proportional relationship of the response to the analyte concentrations over the working range. Peak-area ratios of suxibuzone, phenylbutazone and oxyphenbutazone to the in-

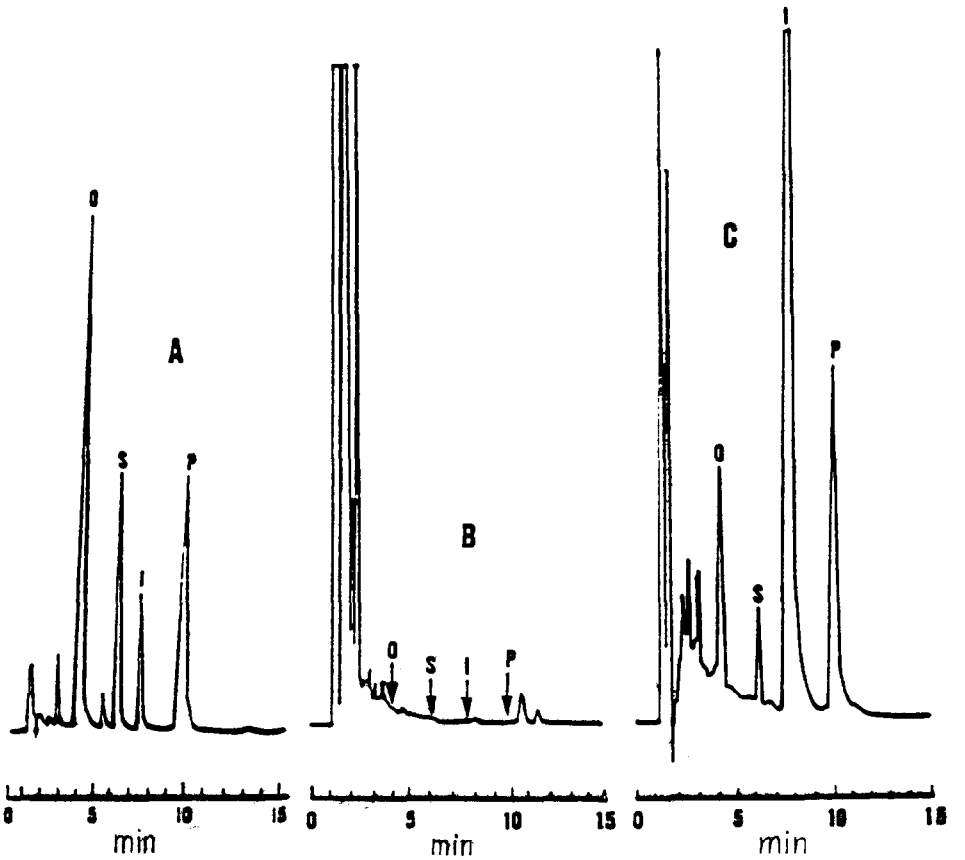

Fig. 1. Chromatograms obtained from (A) human plasma spiked with 10 μg/ml each of oxyphenbutazone, suxibuzone and phenylbutazone and 4 μg/ml indomethacin, (B) racehorse plasma, found negative in doping control, and (C) racehorse plasma found positive; found: 0.9 μg/ml suxibuzone, 0.5 μg/ml oxyphenbutazone and 1.5 μg/ml phenylbutazone. Peaks: O = oxyphenbutazone; S = suxibuzone; I = indomethacin; P = phenylbutazone.

ternal standard were plotted against concentrations and subjected to least-squares regression analysis.

The linearity was determined in the concentration range 0.05–50 μg/ml. For suxibuzone, it was $y = 0.176x - 0.049$ ($r = 0.9996$), for phenylbutazone it was $y = 0.248x + 0.0317$ ($r = 0.9998$) and for oxyphenbutazone it was $y = 0.235x + 0.0890$ ($r = 0.9986$).

The limit of quantitation, defined as the lowest concentration that could be determined with a coefficient of variation (C.V.) less than 15%, was *ca.* 0.05 μg/ml for all three substances.

Precision and accuracy

The intra-day precision, expressed by the C.V., was determined for suxibuzone, phenylbutazone and oxyphenbutazone at three different concentrations. The values ($n = 5$) were 0.6–6.1% for suxibuzone, 0.4–5.9% for phenylbutazone and 0.4–5.4% for oxyphenbutazone. The day-to-day C.V. ranged from 2.8 to 5.9% for suxibuzone, from 2.9 to 3.2% for phenylbutazone and from 2.4 to 6.2% for oxyphenbutazone (Table I).

The accuracy of the method for suxibuzone, phenylbutazone and oxyphenbutazone was evaluated at three different concentrations. The results are shown in Table II.

Selectivity

No interference peaks from plasma were observed. The selectivity of the solid–liquid extraction was compared with that of two liquid–liquid extraction methods: (1) plasma brought to pH

TABLE I

INTRA- AND INTER-DAY PRECISION OF THE ASSAY FOR DETERMINATION OF SUXIBUZONE, PHENYLBUTAZONE AND OXYPHENBUTAZONE IN HUMAN PLASMA

Concentration (μg/ml)	Coefficient of variation (%)		
	Suxibuzone	Phenylbutazone	Oxyphenbutazone
Intra-day precision (n = 5)			
0.1	5.1	5.9	4.6
1.0	6.1	2.5	5.4
10	0.6	0.4	0.4
Inter-day precision (n = 5)			
1	5.9	3.2	6.2
10	2.8	2.9	2.4

3.4 with citrate buffer and extracted by shaking with hexane–diethyl ether (1:1, v/v); and (2) plasma brought to pH 2 with hydrochloric acid and extracted with the same solvent (Fig. 2).

Recovery

The recoveries for the three compounds at four different concentrations were calculated by comparing extracted standard samples with unextracted standards, which represented 100% recovery (Table III).

Degradation products

A suitable solvent for the extraction of suxibuzone, phenylbutazone, oxyphenbutazone and indomethacin (internal standard) was investigated. Owing to the differences in polarity between the substances and the risk of degradation, several organic solvents were tested and the influence of the acidity was studied (Table IV). No degradation was observed for suxibuzone or indomethacin, but phenylbutazone and oxyphenbutazone were observed to degrade (Fig. 3B).

Stability

Phenylbutazone and oxyphenbutazone methanolic solutions degraded during storage at 4°C (Fig. 3A). New solutions were prepared every week.

TABLE II

ACCURACY OF SUXIBUZONE, PHENYLBUTAZONE AND OXYPHENBUTAZONE ASSAY

Real value (μg/ml)	Suxibuzone		Phenylbutazone		Oxyphenbutazone	
	Observed value (μg/ml)	Relative error (%)	Observed value (μg/ml)	Relative error (%)	Observed value (μg/ml)	Relative error (%)
0.150	0.160	6.7	0.152	1.3	0.155	3.3
0.600	0.570	5.0	0.557	7.2	0.588	2.0
1.500	1.440	4.0	1.399	6.7	1.490	0.7

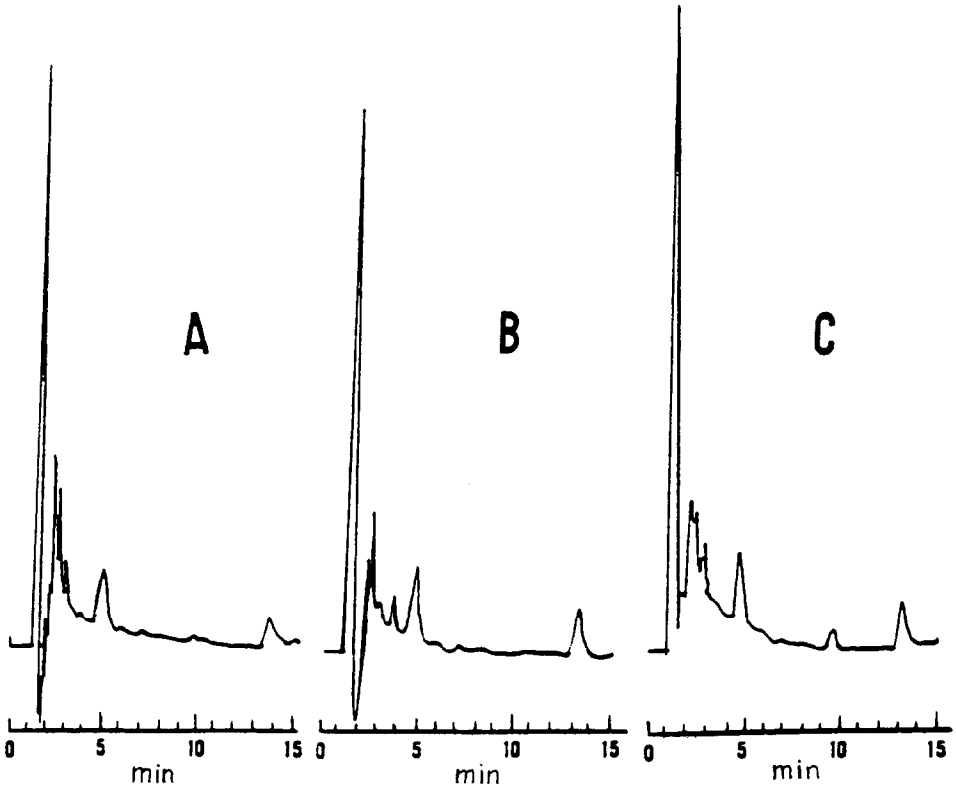

Fig. 2. Chromatograms obtained from extracted blank human plasma. (A) Solid-phase extraction; (B) hexane–diethyl ether (1:1, v/v) with citrate buffer (pH 3.4); (C) hexane–diethyl ether (1:1, v/v) with 5 *M* HCl (pH 2).

DISCUSSION

A problem usually not taken into account during the development of a quantitative HPLC method for phenylbutazone is the degradation of this drug and its metabolites not only upon storage, but also on extraction under acidic conditions. Marunaka and co-workers [1,8] used benzene–cyclohexane to extract suxibuzone and its metabolites after acidification with 5 *M* hydrochloric acid. We tested different organic extraction solvents and acidities, and we observed that degradation of phenylbutazone and oxyphenbutazone took place when 1–5 *M* hydrochloric acid

TABLE III

RECOVERY OF SUXIBUZONE, PHENYLBUTAZONE AND OXYPHENBUTAZONE FROM HUMAN PLASMA

Concentration (μg/ml)	Recovery (%)		
	Suxibuzone	Phenylbutazone	Oxyphenbutazone
1	92.8	90.8	96.4
5	97.1	97.2	91.3
10	96.0	93.9	94.0
50	99.0	99.5	101.5

TABLE IV

DEGREE OF DEGRADATION AFTER 30 min OBSERVED WITH DIFFERENT EXTRACTION SOLVENTS

Extraction solvent	Observed degradation (%)			
	Oxyphenbutazone	Suxibuzone	Indomethacin	Phenylbutazone
Ethyl acetate, pH 2 with 1 *M* HCl	10–50	No	No	10–50
Hexane–2-propanol (1:1, v/v), pH 2 with 1 *M* HCl	1–10	No	No	10–50
Chloroform, pH 2 with 5 *M* HCl	50–70	No	No	50–70
Benzene–cyclohexane (1:1, v/v), pH 2 with 5 *M* HCl	1–10	No	No	1–10
Hexane–diethyl ether (1:1, v/v), pH 2 with 5 *M* HCl	1–10	No	No	1–10
Hexane–diethyl ether (1:1, v/v), pH 2 with 1 *M* HCl	1–10	No	No	1–10
Hexane–diethyl ether (1:1, v/v), pH 3.4 with 0.345 *M* citrate buffer	0–1	No	No	0–1

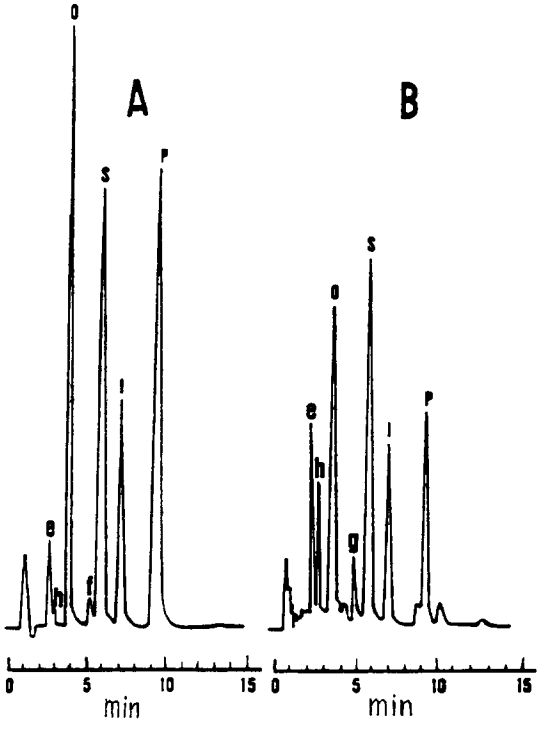

Fig. 3. (A) Chromatogram obtained from a methanolic 10 μg/ml solution of the standards after standing for one week at 4°C (4 μg/ml indomethacin); peaks e and h correspond to oxyphenbutazone degradation and peak f corresponds to phenylbutazone degradation. (B) Chromatogram obtained from a 0.5-ml human plasma sample spiked with 5 μg each of oxyphenbutazone (O), suxibuzone (S) and phenylbutazone (P) and 2 μg of indomethacin (I) and extracted with hexane–diethyl ether (pH 2) with 5 *M* HCl. Peaks e and h correspond to oxyphenbutazone degradation and peak g corresponds to phenylbutazone degradation.

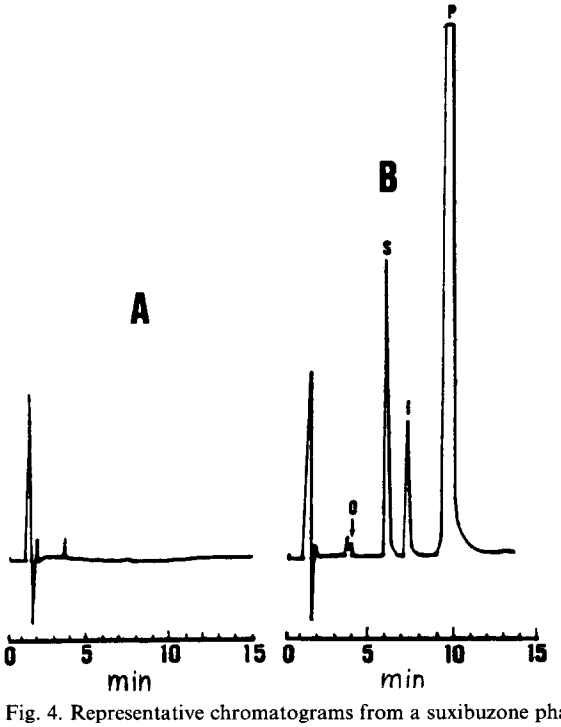

Fig. 4. Representative chromatograms from a suxibuzone pharmacokinetic study in a calf. (A) Drug-free calf plasma; (B) calf plasma sample obtained 3 h after an i.m. dose of 15 mg/kg suxibuzone. Found: 6.7 μg/ml suxibuzone, 30.6 μg/ml phenylbutazone and 0.07 μg/ml oxyphenbutazone.

was used to acidify the samples. To avoid this acidic degradation Perego *et al.* [2] used *n*-heptane to extract their samples after acidification with 1 *M* hydrochloric acid, but they found it was necessary to dry the organic layer over Na_2SO_4. Similarly, Franssen *et al.* [12] used 0.345 *M* citrate buffer (pH 2.0) and neutralized traces of acid with solid $NaHCO_3$.

The main advantages of solid-phase extraction are first that it prevents degradation of the compounds, in a one-step extraction procedure with high recovery, and secondly that it improves the selectivity, as proved by the lack of endogenous interference. The isocratic elution system used gave good separation of indomethacin, suxibuzone, its metabolites and degradation products.

The present method was successfully applied in pharmacokinetic studies on suxibuzone (Fig. 4) and racehorse plasma doping control samples (Fig. 1).

CONCLUSION

The present HPLC method is isocratic and sensitive and has improved selectivity. The sample preparation procedure avoids degradation of the compounds and allows the automation of the analysis of large numbers of samples. It is useful for pharmacokinetic studies, drug monitoring or doping control of these substances in plasma.

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