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Note

Rapid and sensitive high-performance liquid chromatographic assay for metformin in plasma and urine using ion-pair extraction techniques

JULIE KEAL and ANDREW SOMOGYI*

Department of Clinical and Experimental Pharmacology, University of Adelaide, G.P.O. Box 498, Adelaide 5001 (Australia)

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Metformin (1,1-dimethylbiguanide) is an hypoglycaemic, biguanide drug clinically used in the treatment of non-insulin-dependent diabetes. Information concerning the mechanisms and magnitude of gastrointestinal absorption, distribution and elimination of metformin in man is scant. This is mainly because of the difficulty in measuring low enough concentrations of the drug in biological fluids for pharmacokinetic analyses. In particular, metformin is highly polar (octanol:water = 0.01) and is therefore extremely difficult to extract from biological fluids.

Two published gas chromatographic (GC) assays used a complex derivatization procedure and subsequent electron-capture detection to enable the estimation of metformin in biological fluids with a lower limit of sensitivity of 1 — 2 ng/ml [1, 2]. A high-performance liquid chromatographic (HPLC) assay was reported for urine analysis of metformin [3] in which samples were derivatised prior to injection onto the column. It was applicable to urine only, with a lower limit of sensitivity of 200 ng/ml. This method has been adapted to measure metformin in dog plasma [4]. Although of adequate sensitivity it required a derivatization procedure and GC using a nitrogen detector. A recent HPLC assay using protein precipitation of plasma with subsequent injection of the supernatant onto a cation-exchange HPLC column claimed a sensitivity limit of between 50 and 100 ng/ml [5]. Our experience with this assay was unfruitful, as a number of endogenous substances interfered with the detection of metformin.

Prior to the publication of these assay methods, Garrett and co-workers [6, 7] reported on an ion-pair method for the extraction of biguanides (phen-

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formin, metformin and buformin) from biological fluids with subsequent spectrophotometric detection. The limit of sensitivity was about 1 μg/ml from 4 ml of plasma, and the assay required large volumes of sample and reagents. We report here on a modification of the Garrett method [6, 7], which is simpler, more sensitive (to 0.01 μg/ml), uses HPLC with UV detection, readily lends itself to assaying large numbers of samples and has enabled investigations into the pharmacokinetics of metformin in patients.

EXPERIMENTAL

Reagents

All reagents were of analytical grade and included diethyl ether and orthophosphoric acid (BDH Chemicals, Port Fairy, Australia), dichloromethane (Mallinckrodt, South Oakleigh, Australia), dipotassium hydrogen orthophosphate (Ajax Chemicals, Sydney, Australia), heptane sulphonic acid, bromothymol blue and tetrabutyl ammonium hydroxide (TBAH) (Sigma, St. Louis, MO, U.S.A.). Acetonitrile (Mallinckrodt) was of HPLC grade. Metformin hydrochloride was supplied by Lipha Pharmaceuticals (West Drayton, U.K.); the internal standard, 1-propylbiguanide sulphate was generously provided by Dr. Peter Ravenscroft (Department of Clinical Pharmacology, Princess Alexandra Hospital, Brisbane, Australia).

Apparatus

The high-performance liquid chromatograph consisted of an SP8770 isocratic pump (Spectra-Physics, San Jose, CA, U.S.A.), a Jasco Uvidec 100V variable-wavelength detector (Japan Spectroscopic Co., Tokyo, Japan), a Waters WISP 710B automatic injector (Waters Assoc., Milford, MA, U.S.A.), and an Omniscribe B-5000 strip-chart recorder (Houston Instruments, Austin, TX, U.S.A.). The 15 cm × 4.6 mm I.D. stainless-steel column was homepacked with Spherisorb 5-μm ODS 2 packing material batch 22/133 (Phase Separations, Queensferry, U.K.). The mobile phase was 8% acetonitrile, 3 m*M* heptane sulphonic acid, 0.05 *M* dipotassium hydrogen orthophosphate, and distilled water to 100%, adjusted to pH 4.0 with orthophosphoric acid. The flow-rate through the column, at ambient temperature, was 1 ml/min, which produced a back-pressure of 12.1 MPa. The detector wavelength was set at 234 nm, and peak heights were measured.

Stock solutions

Metformin was made up as a 1 mg/ml stock solution in methanol and was diluted to concentrations ranging from 0.01 to 2.0 μg/ml in blank plasma and from 25 to 300 μg/ml in blank urine. Propyl biguanide, internal standard stock solution (1 mg/ml in methanol), was diluted with distilled water to concentrations of 0.1 μg per 50 μl for plasma analysis and 2.0 μg per 100 μl for urine analysis. Bromothymol blue (0.01 *M*) was prepared by dissolving 1.48 g bromothymol blue in 20 ml of 0.2 *M* sodium hydroxide and adjusting the final volume to 200 ml with distilled water. The solution was left standing at room temperature (with occasional mixing) for two days before use. For plasma analysis, the TBAH concentrated solution was diluted to 0.1% with distilled

water and for urine analysis to 1.0%, both of which were adjusted to pH 7.0 by the addition of a few drops of 0.1% orthophosphoric acid.

Sample preparation

Plasma. For metformin concentrations in the range 0.01—0.1 μg/ml, 1 ml of plasma was pipetted into a 10-ml screw-capped, tapered plastic tube (Mallinckrodt) to which were added 0.25 ml water. For concentrations greater than 0.1 μg/ml, 0.25 ml of plasma and 1 ml of distilled water were used. To these were added 1 ml of 0.01 *M* bromothymol blue and 50 μl containing 0.1 μg of internal standard. The mixture was vortexed briefly, and then the pH was adjusted to 7.6—7.8 by the addition of a few drops of dilute (0.1%) orthophosphoric acid. Then, 5 ml of a mixture of diethyl ether- dichloromethane (2:1) were added and the tubes were vortex-extracted for 1 min. The organic and aqueous phases were separated by centrifugation at 2000 *g* for 5 min. A 4-ml volume of the upper organic phase was transferred to a clean 10-ml screw-capped, tapered plastic tube containing 100 μl of 0.1% TBAH in water. The tubes were vortex-mixed for 1 min and the two phases separated by centrifugation at 2000 *g* for 5 min. The organic layer was discarded and the tubes were then placed in hot water (70—90° C) for 10 min to remove all trace of organic solvents. An aliquot (usually 25 μl) was injected onto the column via the automatic injector.

Urine. A 20-μl aliquot of urine was pipetted into the 10-ml screw-capped, tapered plastic tube, and 2.0 ml of 0.01 *M* bromothymol blue and 100 μl of the urine internal standard solution were added. The samples were then handled in the same manner as for the plasma samples, except that 100 μl of 1.0% TBAH were used.

RESULTS AND DISCUSSION

Fig. 1 shows a chromatogram of an injection of a standard solution of metformin and propylbiguanide. Metformin had a retention time of 3.3 min and a capacity factor of 0.83, whereas propyl biguanide had a retention time of 7.80 min and a capacity factor of 3.33. Calibration curves in plasma and urine showed good linearity between peak-height ratios and concentration from 0.01 to 0.1 μg/ml (r^2 = 0.993, 95% confidence interval of slope = 9.8%) and 0.1 to 2.0 μg/ml (r^2 = 0.999, 95% confidence interval of slope = 5.3%) in plasma and from 25 to 300 μg/ml (r^2 = 0.999, 95% confidence interval of slope = 6.4%) in urine. For both plasma and urine standard curves, the 95% confidence intervals of the intercepts included the origin. The accuracy of the method at a plasma concentration of 0.05 μg/ml was ± 0.004 μg/ml and at 0.5 μg/ml it was ± 0.02 μg/ml. In urine at a concentration of 200 μg/ml the accuracy was ± 6 μg/ml. Fig. 2 shows a chromatogram from blank plasma, a plasma sample from a subject 3 h after ingestion of 250 mg of metformin and a urine sample from a subject during a 24-h dosing interval.

The assay showed good precision at low and high metformin concentrations in both plasma and urine. Table I shows the intra- and inter-day assay variability. The limit of determination was 0.01 μg/ml defined as four times the baseline noise level. Using this method the recovery of metformin from plasma

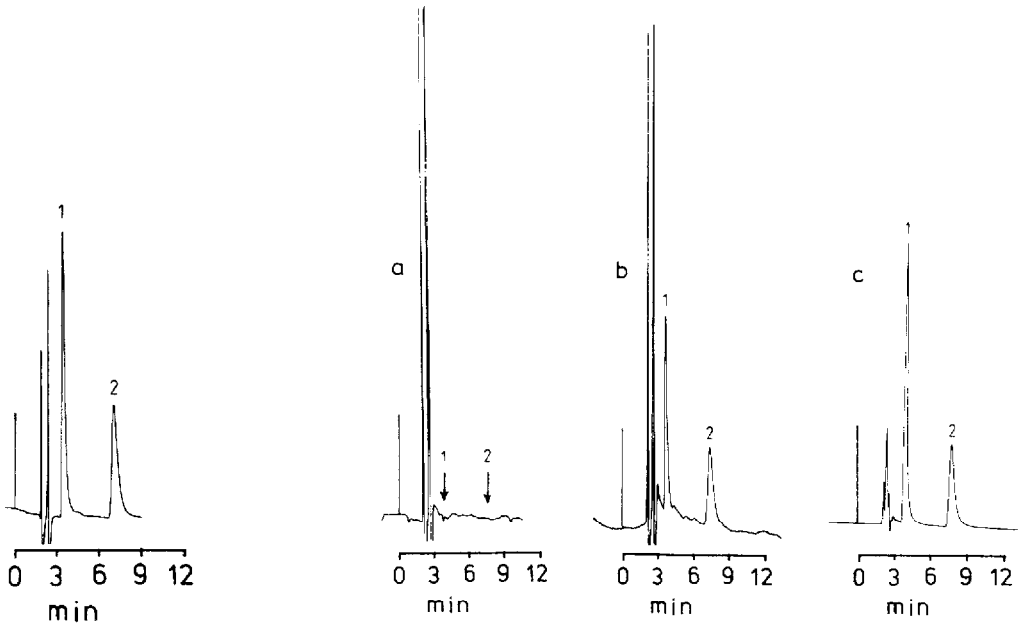

Fig. 1. Chromatograms of an injection of a standard solution of metformin (1) and propylbiguanide, internal standard (2) (100 ng of each onto the column).

Fig. 2. Chromatogram from an extract of (a) a blank plasma sample, (b) a plasma sample from a subject 3 h after ingestion of 250 mg of metformin (metformin concentration was 0.984 μg/ml) and (c) a urine sample from a subject during a 24-h dosing interval (metformin concentration was 189 μg/ml). Peaks: 1 = metformin; 2 = propylbiguanide, internal standard.

TABLE I

ASSAY VARIABILITY OF METFORMIN IN PLASMA AND URINE

Sample	Concentration (μg/ml)	Coefficient of variation (%)	*n*
Intra-day			
Plasma	0.1	4.84	7
	1.0	2.54	8
Urine	50	6.32	7
	300	4.32	8
Inter-day			
Plasma	0.75	4.74	7
Urine	150	3.30	4

averaged 27.5% and for propylbiguanide 78.7%. A double extraction of plasma samples with the organic mixture resulted in an improved metformin extraction of 43 and 98% for the internal standard. Some plasma samples occasionally showed endogenous peaks that eluted between metformin and the internal standard. These were readily resolved from the metformin peak by lowering the acetonitrile concentration (by 1%) in the mobile phase. Plasma samples stored at −10°C for at least five months showed no decline in metformin concentration.

The method of extraction described here is derived from the original studies of Schill and co-workers [8, 9] in which the concentration of bromothymol blue and the pH for maximum ion-pair formation is determined by the physico-chemical characteristics and concentration of the organic cation, in this case metformin. The optimal conditions for metformin were derived by a series of experiments. The organic mixture of dichloromethane and diethyl ether resulted in a recovery of metformin similar to that achieved by Garrett et al. [7] in which only dichloromethane was used. Although the recovery was low, it showed only a small degree of variability and was deemed acceptable considering the low concentrations which could be measured and the small inter- and intra-day assay variations. Diethyl ether by itself resulted in only 6% extraction of metformin and was used mainly as a carrier so that the organic phase was the upper layer, hence enabling greater ease of handling.

The selection of TBAH as used by Garrett and co-workers [7, 8] was found to be quite critical. Tetrabutyl ammonium phosphate (Sigma; PIC Reagent A, Waters Assoc.), tetrabutyl ammonium hydrogen sulphate (Sigma), tetrabutyl ammonium bromide and tetramethyl ammonium iodide (L. Light & Co., Colnbrook, U.K.), although suitable counter cations to metformin, all showed spurious and interfering peaks when injected on the chromatography system.

This assay is a unique application of the ion-pairing extraction method reported by Schill and co-workers [8, 9] and Garrett and co-workers [6, 7]. Its subsequent use of HPLC enables precision, sensitivity and automation (using the WISP automatic injector), and is an improvement on previously published assays, in that complex derivatisation procedures are not necessary. The advantage of a C_{18} reversed-phase column over a cation-exchange column greatly reduces the costs and increases the life-time of the column: for example, over 1000 samples have been injected onto the column without significant loss of column efficiency. In a normal working day one person can prepare 48 samples (including standards) for overnight automatic injection.

This assay has enabled investigations into the pharmacokinetics of metformin and renal drug interactions between metformin and cimetidine [10]. It may be valuable in the therapeutic monitoring of metformin in the plasma of diabetic patients.

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