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Note

Assay of trimethoprim in plasma and urine by high-performance liquid chromatography using electrochemical detection

LARS NORDHOLM* and LARS DALGAARD

Royal Danish School of Pharmacy, Department of Chemistry BC, 2 Universitetsparken, DK-2100 Copenhagen (Denmark)

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Trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine] (TMP) is a synthetic antibacterial, which interferes with folic acid metabolism by inhibiting dihydrofolate reductase (EC 1.5.1.3) [1]. The drug is administered either alone or in combination with a sulphonamide.

Various analytical methods for quantitation of TMP in body fluids have been used, including microbiological assay [2], spectrofluorimetry [3], autoradiography [4], differential pulse polarography [5], thin-layer chromatography with densitometry [6], and gas—liquid chromatography [7].

Several high-performance liquid chromatographic (HPLC) methods for determination of the drug in biological fluids have appeared [8—15]. Both normal-phase [8,11,15], reversed-phase [9,10,13,14], and ion-pair systems [12] have been employed. Clean-up procedures vary from simple precipitation of proteins [9,10] to extraction with chloroform [8,11], methylene chloride [12] or ethyl acetate [13—15].

The aim of this investigation was to develop a sensitive method, which enables handling of small volumes of plasma samples containing TMP at the 0.1 ppm level. Only small plasma volumes could be provided, as the assay was used in pharmacokinetic studies of TMP in newborn pigs. Existing methods for determination of TMP were not found suitable for measurements at the 0.1 ppm level, which was close to the detection limit using UV detection.

However, the electrochemical detector working in the oxidative mode has proved to be of value in trace analysis of, for example, phenols and aromatic amines [16]. Therefore, it seemed to be a promising alternative to use electrochemical detection (ElCD).

Suitable conditions (e.g. simple extraction procedure, small retention volumes) for use in pharmacokinetic studies involving a large number of samples were elucidated.

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EXPERIMENTAL

Apparatus

HPLC was performed with a Waters Model 6000 solvent delivery system connected to a Waters Model 440 UV detector (280 nm) and an electrochemical detector consisting of a Model 656 electrochemical detector and a Model 641 VA detector (Metrohm, Switzerland). Working electrode: glassy carbon. Reference electrode: Ag/AgCl. Auxiliary electrode: glassy carbon. The detectors were connected in series with the electrochemical detector downstream. The injection port was a Rheodyne Model 7125 loop injector provided with a 100-μl loop. An Omniscribe Model 5111-5 recorder (Houston Instruments, Houston, TX, U.S.A.) was employed. A stainless-steel HPLC column (Knauer, 120 × 4.6 mm I.D.) connected to a 40-mm precolumn of similar type was used. Both columns were packed with Nucleosil C_{18} (5 μm) particles (Macherey-Nagel, Düren, G.F.R.). The mobile phase was 0.07 *M* KH_2PO_4 (pH 4.75)—methanol (3:1). The procedure was carried out at a flow-rate of 1.5 ml/min (200 bars).

Chemicals

TMP was a gift from Syntex, Grindsted, Denmark. Sulphamethoxazole (SMZ) was donated by DAK-laboratories, Copenhagen, Denmark. All other chemicals were of analytical grade.

Standard solutions of TMP and SMZ were made from stock solutions of the compounds in 10% methanol by diluting with mobile phase. For recovery studies TMP solutions were made by diluting the stock solution with 0.1 *M* sodium hydroxide.

Internal standard

During the study SMZ was used as internal standard because chromatographic behaviour of this compound in combination with TMP is well described [9,10,13,14]. SMZ was dissolved in mobile phase and added to the samples after extraction, and thus functioned as a volume marker. Besides, the internal standard could check stability of the electrochemical detector within-day.

Procedure

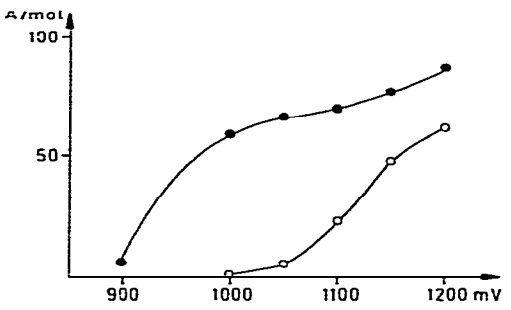
A 250-μl plasma sample together with 50 μl of 0.1 *M* sodium hydroxide and 1500 μl of ethyl acetate was pipetted into a 3-ml polypropylene tube. The tube was stoppered, mixed for 15 sec on a vortex mixer, and centrifuged for 1 min at 18,000 *g*. Then 1000 μl of the organic phase were transferred to another polypropylene tube and evaporated under a stream of nitrogen at 40°C. The residue was redissolved in 250 μl of mobile phase containing internal standard. After mixing for 30 sec on a vortex mixer and centrifugation for 1 min at 18,000 *g*, 10—100 μl were injected into the HPLC system. Urine samples were treated in the same way after diluting ten times with distilled water.

Concentrations of TMP in plasma or urine were measured by comparing peak height ratios of TMP/internal standard with peak height ratios of extracts from

blank plasma/urine samples with known amounts of TMP added. For recovery studies extracted blank plasma/urine samples with known amounts of TMP added were compared with TMP standard solutions.

RESULTS AND DISCUSSION

The response characteristics of the described ElCD system are shown for TMP and internal standard in Fig. 1. It is seen that TMP has not reached its plateau at 1200 mV. Comparison of the sensitivity of ElCD and UV detection of TMP is shown in Fig. 2.



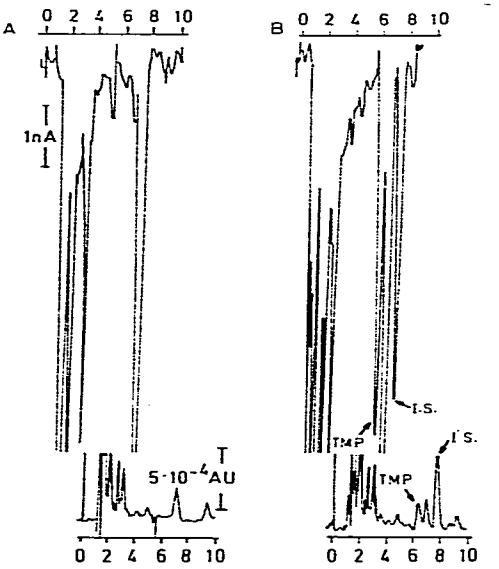


Fig. 1. Applied potential vs. output current. Output current is determined by injecting the same sample [250 ng of TMP (○), 70 ng of sulphamethoxazole (●)] at varying potentials.

Fig. 2. Electrochemical detection at 1200 mV (top) and UV detection at 280 nm (bottom) of (A) blank plasma extract, (B) plasma extract from a 67-day-old pig 7 h after intravenous injection of 5 mg/kg TMP. Concentrations of TMP and internal standard (I.S.) are 0.25 ppm and 0.07 ppm, respectively.

At 1200 mV a linear relationship was found between the concentration of TMP and the ratio of the peak heights of TMP/internal standard in the concentration range studied (1—250 ng on-column sample weight).

The detection limits, i.e. the minimum plasma concentration detectable, defined as twice the baseline noise for UV detection and twice a non-specific long-term noise in the case of ElCD, was found to be 0.01 ppm (ca. 2 pmol injected) using ElCD (1200 mV), and 0.1 ppm using UV detection (280 nm), when extractions were performed as described above.

Recoveries from the extraction procedure are presented in Table I.

When the required pressure for working the system at 1.5 ml/min exceeded 233 bars, the top filter of the precolumn was renewed (after several hundred injections) or, if necessary, the precolumn was repacked.

TABLE I

RECOVERY OF TMP ADDED TO PIG PLASMA AND URINE

The data represent mean ± S.D. for five determinations of samples at each concentration level.

	TMP concentration (ppm)	Recovery (%)
Plasma	0.1	98.1 ± 3.7
	0.5	89.6 ± 2.0
	1.0	93.6 ± 2.2
	5.0	87.9 ± 2.1
Urine	25	83.7 ± 0.4

Employment of the electrochemical detector in HPLC analysis of TMP in plasma has the advantage over UV detection that the sensitivity is enhanced. Working at high potential will often impair the analysis, because many compounds are detected and thus capable of interfering the assay. When analysing TMP, interfering peaks can be avoided by a single extraction with ethyl acetate. The choice of 1200 mV was a compromise between sensitivity and selectivity. Working the glassy carbon electrode above 1200 mV is, furthermore, not recommended by the producer. Besides, the long-term baseline noise was increased by applying a higher potential.

In conclusion, we have found that ElCD at 1200 mV is suitable for determination of TMP in blood plasma and urine, and the sensitivity by using this type of detection is superior to 280-nm UV detection. A minor drawback is a non-specific long-term baseline noise, which is present at high oxidation potentials, while electrode passivation is an unimportant problem which can be overcome by routine repolishing. Actually, the chromatographic system was run for several weeks (30—40 injections daily) before repolishing of the glassy carbon electrode was necessary. The fact that the applied potential was placed on the ascending part of the curve did not cause serious problems. Injection of the same standard several times during the day gave rise to variation in the order of 2—3%.

Though the method was developed with the purpose of studying the pharmacokinetics of TMP in pigs during the first weeks after birth [17], it was also found suitable for analysis of human plasma.

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