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Note

High-performance liquid chromatographic determination in human plasma of a new immunomodulatory agent with a peptidyl-hypoxanthine structure (RM 06)

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N-9-Hypoxanthine derivatives are a newly synthesized class of drugs whose immunomodulatory properties have been partially defined in vitro and in various experimental animal models [1–5]. In an attempt to reach a better understanding of the structure–activity relationships within this family of drugs, several compounds have been designed and synthesized by conjugating different oligopeptides to the purine moiety structure of the first-generation drugs [6]. Among these peptidyl derivatives, the compound N-ω-5-[1,6-(dihydro-6-oxo-9-purinyl)pentyloxycarbonyl]-L-leucine-L-methionine (designated RM 06, see Fig. 1) significantly stimulates natural killer cell (NK) activity in lethally irradiated mice reconstituted with syngeneic bone marrow transplant after lethal irradiation [7]. As an immunotherapeutic counterpart, RM 06 significantly reduces the number of lung metastases in lethally irradiated and bone-marrow-reconstituted mice challenged intravenously with B 16 melanoma cells. This anti-metastatic effect is possibly dependent on NK antitumour activity in these immunodeficient animal models [7].

Immune system responses to drugs as well as to naturally occurring biological response modifiers are often strictly dose-dependent, showing a broad quantitative and qualitative spectrum of dose–effect relationships. For this reason, a method for the reliable measurement of these drugs in biological fluids should be available, even in preclinical studies, mainly to allow the cor-
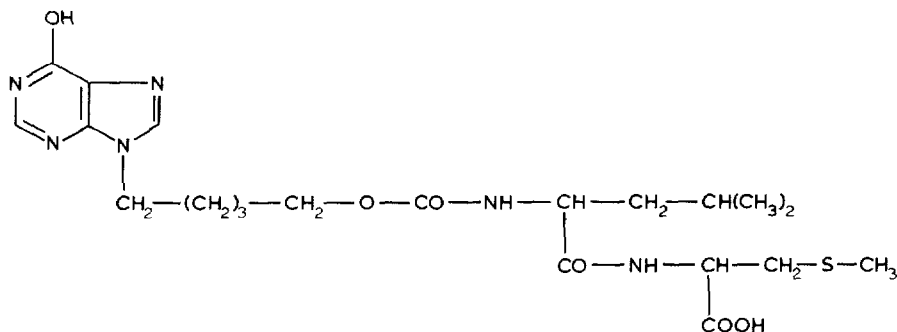
Fig. 1. Structure of RM 06.

rect interpretation of experimental results. Moreover, this would establish a basis for the careful monitoring of plasma drug levels and thorough pharmacokinetic knowledge in clinical settings, where the safe and effective handling of the drug is of utmost importance.

To this purpose we have developed a method for the detection and quantitation of RM 06 in human plasma, based on liquid–solid extraction and reversed-phase high-performance liquid chromatographic (HPLC) analysis.

EXPERIMENTAL

Chemicals

RM 06 and RM 05 (another peptidylhypoxanthine derivative with an L-prolyl-L-arginine moiety, used as internal standard in this study), were a generous gift from Co-Pharma (Cagliari, Italy). Water was HPLC grade obtained from a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.). Acetonitrile was HPLC grade from Merck (Darmstadt, F.R.G.). Analytical-grade monobasic potassium phosphate was from Riedel-De Haan (Hannover, F.R.G.).

Stock solutions

Stock solutions of RM 06 and RM 05 were freshly prepared every three days in water at a concentration of 1 mg/ml and kept at $+4°C$ overnight or in an ice-bath, and light-protected during use. The stability of these solutions was checked daily before use by repeated HPLC analysis and peak absorbance measurement.

Chromatographic apparatus

The system consisted of a Model 501 HPLC pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model 7010 injector (Rheodyne, Cotati, CA, U.S.A.) with a 250-μl loop and a Model 2140 (LKB, Bromma, Sweden) diode array UV detector. A Spherisorb 5 ODS (250 mm×4.6 mm I.D.) column

(Chrompack, Middelburg, The Netherlands) and a 25 mm × 4 mm I.D. cartridge C_{18} guard column (Merck) were used throughout. Absorbance data were evaluated by the Wavescan 2140-201 program (LKB) on an IBM personal computer.

Chromatographic conditions

Standards and samples were analysed isocratically with a mobile phase of 7.35 mM KH_2PO_4–acetonitrile (80:20, v/v) at a flow-rate of 1.0 ml/min. Spectral data were recorded between 220 and 280 nm and showed an absorbance maximum at 250 nm for both RM 05 and RM 06 (data not shown).

Standard curves

Standards for calibration curves were prepared daily by diluting the stock solution of RM 06 with water to final concentrations of 0.156, 0.313, 0.625, 1.25, 2.5 and 5 μg/ml. Volumes of 250 μl of each solution were injected at least three times a day (i.e. at the beginning and at the end of the experiment and one or more times during it).

Sample preparation

Human plasma was obtained from five healthy adult blood donors at the blood transfusion centre in our hospital. Blood was drawn into heparin-containing tubes and centrifuged at 200 g for 10 min. Plasma was immediately transferred to plastic tubes to prevent the release of UV-interfering substances from platelets and red blood cells [8] and frozen at −20°C or used immediately. Plasma (2 ml) was processed without added drug (blank), and aliquots of 2 ml were spiked with RM 06 to the following final concentrations: 0.156, 0.31, 0.625, 1.25 and 2.5 μg/ml.

Plasma samples were passed through a Sep-Pak C_{18} extraction column (Waters Assoc.), previously washed with 5 ml of acetonitrile and 5 ml of water. The column was washed with 5 ml of water and 2 ml of 5% acetonitrile in water, and plasma components were eluted with 1 ml of water–acetonitrile (80:20, v/v) and collected in an Eppendorf tube. Air was flushed through the column to complete drying and to recover exactly a 1-ml volume of eluate. To this, 2.5 μl of a 1 mg/ml solution of RM 05 (internal standard) was added (the internal standard in this case served only to correct for slight unevenness in loop-filling between injections).

Data analysis

Peaks were quantitated by direct reading on the computer of the absorbance at the peak apex, and by adding or subtracting the absorbance value at the baseline whenever it differed from zero.

The validity of the method was established in two ways:

(a) Linearity of the concentration–absorbance relationship for both the

standard aqueous solutions and the plasma samples: this was accomplished by linear regression analysis of the peak absorbance versus concentration (for aqueous standards) or the ratio of RM 06/internal standard absorbances versus the nominal concentration of RM 06 in plasma.

(b) Extraction efficiency of the sample preparation: this was done by calculating the percentage recovery in plasma samples by the equation:

$$\text{recovery } (\%) = \frac{\text{absorbance of RM 06 in plasma sample}}{\text{absorbance of the corresponding aqueous standard}} \times 100$$

Since 2 ml of plasma were extracted for each dosage, and the same amount (250 μl) of extracted sample and aqueous standard was injected, this recovery was evaluated by dividing the peak of RM 06 in each plasma sample by the peak in the aqueous standard, which had a concentration double that in plasma.

RESULTS AND DISCUSSION

Standard curves

Standard curves with RM 06 concentrations in the range 0.156–5 μg/ml were prepared daily, and injections for each point (number of points $= 6$) were made at least three times a day. The straight lines obtained by plotting the peak absorbance versus RM 06 concentration in aqueous standards had always correlation coefficients greater than 0.999 ($p < 0.01$). The lowest amount detectable by this model was ca. 40 ng (assuming a signal-to-noise ratio of 3), corresponding to a concentration of ca. 0.15 μg/ml (data not shown).

Chromatographic profile

Fig. 2 shows chromatograms of plasma samples containing (A) no RM 06 or internal standard (blank), (B) RM 06 (0.625 μg/ml) and internal standard (RM 05, 2.5 μg/ml) and (C) RM 06 (1.25 μg/ml) and the same amount of internal standard as in B. A 250-μl aliquot of the 1-ml final volume of extracted material was injected. In the blank plasma a slight deflection from the baseline is observed when the internal standard elutes, but it does not significantly interfere with the measurement of the RM 05 peak. On the other hand, RM 06 elutes in a very 'clean' portion of the chromatogram where no disturbing peaks are present in the blank plasma sample. This fact allows very small amounts of the drug to be detected (detection limit ca. 40 ng). The peak eluting between the internal standard and RM 06 (retention time ca. 7.5 min) has not been identified; its presence in the blank plasma, however, rules out the possibility that it is due to some product of rapid degradation of RM 06 or RM 05. Another peak, eluting at ca. 4.5 min, was observed both in blank plasma and in drug-spiked samples; the peak absorbance of this compound varied broadly from sample to sample (even in plasma from the same donor) and was totally unrelated to the presence and concentration of RM 06 and internal standard, so
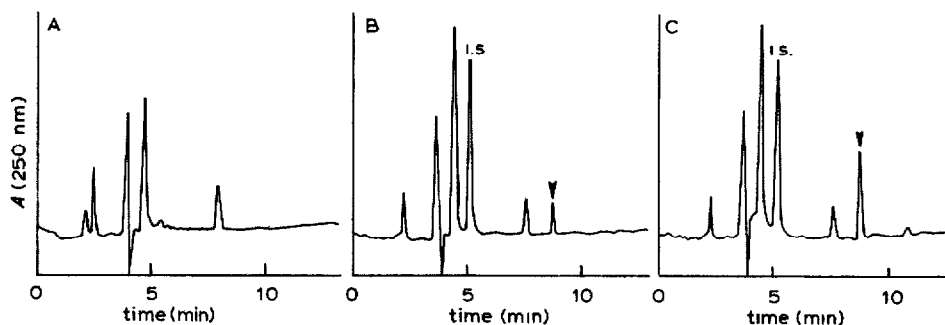
Fig. 2. Chromatographic profiles of (A) blank plasma, (B) plasma containing RM 06 (0.625 μg/ml) and internal standard (i.s.) (RM 05, 2.5 μg/ml) and (C) plasma containing RM 06 (1.25 μg/ml) and the same amount of internal standard as in B. Eluent, 7.25 mM KH$_2$PO$_4$–acetonitrile (80:20, v/v); flow-rate, 1.0 ml/min; detection, UV at 250 nm; sensitivity, 0.042 a.u.f.s. The RM 06 peak is arrowed.

TABLE I

ANALYTICAL RECOVERY OF RM 06

RM 06 was extracted and chromatographed as described in the text.

Concentration added (μg/ml)	Concentration recovered (mean $\pm$ S.D., $n=5$) (μg/ml)	Recovery (%)
0.156	0.139 $\pm$ 0.010	89 $\pm$ 7
0.313	0.267 $\pm$ 0.025	86 $\pm$ 8
0.625	0.562 $\pm$ 0.012	90 $\pm$ 2
1.25	1.225 $\pm$ 0.075	98 $\pm$ 6
2.5	2.325 $\pm$ 0.15	93 $\pm$ 6

it is likely to represent an endogenous compound with very variable extraction recovery.

Plasma extraction and drug recovery

Table I shows the mean percentage recovery ($\pm$ S.D.) achieved in five different experiments using plasma from different donors. The recovery ranged from 86 to 98%, depending on the dose. A reproducible result was the almost quantitative recovery only at a plasma drug concentration of 1.25 μg/ml. A possible explanation of this odd observation is that concentration-dependent phenomena of aggregation between the relatively non-polar molecules of this drug occur, rendering them more or less available for the binding to the hydrophobic phase of the C$_{18}$ extraction cartridge. If this is the case, however, a non-linear relationship of this self-aggregating process with the drug concentration

must be assumed, in view of the fact that recovery at concentrations higher than 1.25 μg/ml is similar to that for lower concentrations.

Plasma concentration–absorbance linearity

The best-fit straight line obtained by plotting the ratio of RM 06 peak absorbance to that of the internal standard versus the drug concentration in plasma (mean of five experiments) is described by the regression equation $y = 0.0246 + 0.2724x$ with a correlation coefficient of 0.99182 ($p < 0.01$).

CONCLUSION

We present a method for the detection and quantitative evaluation in human plasma of one member of a very promising family of compounds with ascertained immunostimulatory activity [1–6]; for some of these drugs, moreover, a striking stimulatory activity on murine and human myelo- and monocytic bone marrow precursors has been observed [10].

As with other drugs and/or physiologically produced factors active upon the immune system and bone marrow cells, a broad variability in the amount and quality of effects is to be expected, depending on the drug concentration at the site of action. The possibility of measuring the drug concentration and keeping it within the desired range would probably improve both our comprehension of the pharmacological effects and, consequently, the rationale for use of the drug for experimental (and possibly clinical) purposes. As far as methodological aspects are concerned, the procedure presented here is simple and rapid to perform. It involves a liquid–solid extraction step exploiting the relative hydrophobicity of the peptidyl moiety of the drug (L-Leu-L-Met), followed by a 10-min reversed-phase HPLC analysis. However, since no information is currently available regarding the in vivo kinetics and metabolism of RM 06 in either experimental animals or humans, we still do not know whether this method will prove sufficiently sensitive for pharmacokinetic investigations. Further technical improvements in the detection of this molecule (i.e. coulometric or electrochemical detection) are currently under investigation in our laboratory.

To our knowledge, the present report represents the first contribution to the development of a general strategy for the isolation, identification and quantitative assessment of peptidylhypoxanthine-derived drugs in biological fluids.

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