Differential Inhibitory Effects of Isosorbide Dinitrate and its Mononitrate Metabolites on Platelet Aggregation and Thromboxane Formation

Pierre H. Rolland[1,2], José Sampol[1], Bruno Lacarelle[1], Dominique Arnoux[1], François Leca[1], Elizabeth Gueydon[1], and Jean-Paul Cano[1]

Received: July 26, 1984; accepted: October 4, 1984.

Abstract: The effects of isosorbide dinitrate (ISDN) and its 2- and 5-mononitrate metabolites (2-ISMN and 5-ISMN) against platelet aggregation and thromboxane release were investigated by analysis of platelet aggregation curves. ISDN, 2-ISMN and 5-ISMN (isosorbide nitrates, ISN) inhibited both ADP- and epinephrine (EPI)-induced platelet aggregation. ISN affected specifically the extent of ADP-induced aggregation and the velocity of EPI-induced effects. 2-ISMN was more potent against platelet aggregation compared to ISDN and 5-ISMN. The isosorbide nitrates were poor inhibitors of both arachidonic acid-induced aggregation and platelet TxB2 release. The differential inhibition by the three isosorbide nitrates of endogenous TxB2 release during ADP-induced aggregation further indicates that 2-ISMN is a significantly more potent platelet inhibitor than either ISDN or 5-ISMN. These studies suggest a role of the metabolites in modulating the pharmacological effects of ISDN on platelet activity.

The usual clinical manifestations of coronary-heart diseases are thought to result from vasospastic and thrombotic events, although the exact relationships between coronary spasm, coronary thrombosis, platelet-induced thromboembolism, and myocardial infarction are still under debate (1–5). Organic nitrates are widely used as vasodilators in the treatment of coronary heart disease, but the precise molecular mechanisms by which they act are uncertain (6, 7). In addition to their vasodilating properties, nitroglycerin, nitroprusside and isosorbide dinitrate (ISDN) are inhibitors of platelet aggregation *in vitro* and *in vivo* (8–11, 17). However, there is a marked disparity between concentrations which inhibit platelet aggregation *in vitro* and therapeutic plasma levels obtained *in vivo*, i.e. the mininal *in vitro* inhibitory levels can be several orders of magnitude greater than their *in vivo* counterparts (12, 13). Thus, if these agents inhibit platelet activity *in vivo* when used therapeutically, they must act indirectly. The same mechanism may also be responsible for the vasodilation. Organic nitrates may stimulate the production of a second substance *in vivo* that in turn would decrease vascular tone and inhibit platelet activity. Prostacyclin is a candidate for such a role (11, 14). On the other hand, it is conceivable that the active agent is not the drug itself but rather one or several of its metabolites. These studies were designed to test the latter hypothesis by comparing the inhibitory effects of ISDN and its mononitrate metabolites (2-ISMN and 5-ISMN) on platelet aggregation and thromboxane formation. We report here that 2-ISMN is a more potent inhibitor of platelet aggregation than ISDN and 5-ISMN.

Materials and Methods

Platelets were obtained from normal healthy male or female adult volunteer donors with no evidence of liver or hematological disease and no exposure to aspirin-like drugs or other medication for at least 2 weeks prior to venipuncture. Informed consent was obtained from each subject. Blood was drawn into citric acid/trisodium citrate (0.11 M) buffer, pH 7.4, using a Vacutainer and Hardpack multiple sample needle system, Becton-Dickinson and Co. Platelet-rich plasma (PRP) was prepared by centrifugation of blood at 150 x g for 20 min at 16°C. Platelet-poor plasma (PPP) was prepared by centrifugation of PRP or whole-blood at 6,000 x g for 15 min at 16°C. Washed platelets were obtained from blood drawn into EDTA-Vacutainer tubes by centrifugation of PRP at 4,000 x g for 15 min at 16°C. The pellet was suspended into Ca-free Krebs-Heinseleit buffer containing 5 mM Hepes and was washed twice under the same conditions. The resulting pellet was suspended in the same buffer.

Platelet aggregability was assayed with constant stirring at 1100 rpm in a dualchannel aggregometer (Coultronics) thermostated at 37°C. Platelet suspensions (PS) were prepared immediately before each series of studies by diluting PRP in autologous PPP to counts in the range of 3 to 3.5 × 10^5 platelets per microliter. Platelet counts in fresh PRP were above this range. Prior to the addition of 0.02 ml of aggregating agent solution, a 0.05 ml solution of the agent to be tested was added to 0.4 ml of PS in the aggregometer. In controls, the corresponding buffers were added. The results are given in terms of the change in light transmission (LT) expressed as a percentage of the difference in LT between PRP and PPP once a plateau was reached, thus defining the extent of the aggregation response. The slope of the aggregation response, defining the velocity, was the initial slope since thereafter platelet aggregation slowly reaches a plateau. Therefore, the velocity was the percentage of the difference in LT between PRP and PPP, thirty secondes after the addition of the aggregating agent.

Thromboxane (Tx) production by platelets was measured by specific RIA of TxB2 as previously described (11, 16–18). Washed platelet suspensions (WPS) were diluted to 5 × 10^8 platelets/ml in Krebs-Heinseleit buffer containing 5 mM Hepes, final pH 7.4. Prior to the addition of 0.05 ml of aggregating agent solution, a 0.05 ml solution of the chemicals to be tested at the appropriate concentration was added to 0.4 ml of WPS. In controls, the corresponding buffers were added. Routinely, incubation time

[1] Département de Pharmacologie Cellulaire, Inserm Sc 16, Faculté de Pharmacie, Marseille.

[2] Correspondence to be addressed to P. H. Rolland, Département de Pharmacologie Cellulaire, Inserm Sc 16, Faculté de Pharmacie, F-13385 Marseille Cedex 5, France.

Abbreviations:
ISN, isosorbide nitrates; ISDN, isosorbide dinitrate; 2-ISMN, 2-isosorbide mononitrate; 5-ISMN, 5-isosorbide mononitrate; PS, platelet suspension; LT, light transmission; WPS, washed platelet suspension; EPI, epinephrine; ARA, arachidonic acid.

was 5 min at 37°C in a shaking waterbath. Incubations were terminated by addition of 0.2 ml of citric acid solution (1M), and radioactive TxB2 was added in trace amounts to evaluate procedural losses. After diethyl-ether extraction, the dried organic extracts were submitted to silicic acid chromatography. TxB2 was eluted from the column by 7 ml of benzene : ethyl acetate : methanol (6:4:0.3, vol/vol). RIAs were performed on buffer reconstituted residues from these fractions. Thromboxane production was expressed in nanograms of TxB2 produced by 10^8 platelets/5 min at 37°C (11, 16–18).

The purity of ISDN, 2-ISMN and 5-ISMN standard solution was checked by GLC analysis (30) and was found to be over 98%. Similarly, the final concentration to be tested in these *in vitro* studies were confirmed by GLC.

Results were expressed as means ± standard deviation (SD). Statistical examination was carried out by one-way analysis of variance (ANOVA) or multiple ANOVA (MANOVA).

Results

The basic characteristics of platelet inhibition by 2-ISMN are illustrated by the aggregation curves shown in Fig. 1. Similar results were obtained for the effective concentration of ISDN and 5-ISMN. A marked difference in the inhibiting properties of isosorbide nitrates (ISN) was noted according to the nature of the aggregating agent: ISN lowered the extent but weakly affected the velocity of the aggregation response to ADP, while the reverse situation was observed when EPI was used as the aggregating agent. ISN affected both primary and secondary EPI-induced aggregation in a similar manner. ISN were found to be poor inhibitors of collagen and arachidonate-induced aggregation, since high concentrations (10^{-3} M) were needed to induce a 65% inhibition of platelet aggregation. Fig. 2 shows the effects of ISN on the extent or the velocity of platelet aggregation induced by ADP or epinephrine, respectively. Isosorbide nitrates when used alone are able to elicit an inhibitory response to both EPI- and ADP-induced aggregation. ISDN and 5-ISMN were found to affect platelet aggregation in a similar manner, and high concentrations (10^{-3} M) profoundly affected platelet aggregation. These effects were still statistically significant for 10^{-4} M ISDN or 5-ISMN concentration ($p<0.05$). In the presence of 10^{-5} M ISDN or 5-ISMN, platelet aggregation parameters returned to control levels. By contrast, 2-ISMN was found to be more potent than ISDN and 5-ISMN, since 1) at a concentration of 10^{-3} and 10^{-4} M 2-ISMN completely arrested EPI-induced aggregation and fully reversed ADP-induced aggregation, and 2) at a concentration of 10^{-5} M, 2-ISMN caused a 50% and 15% inhibition of EPI- and ADP-induced aggregation, respectively.

Attempts were made to investigate ISN effects on platelet TxB2 release as an inducer of platelet aggregation, when TxB2 was produced during arachidonic acid (ARA)-induced platelet aggregation, and as a biochemical marker of platelet aggregation, when TxB2 was produced from platelet endogenous arachidonate under exposure to aggregating agents. As shown in Fig. 3, thromboxane B2 production by platelets under ARA-induced aggregation was inhibited by high ISN concentration (5×10^{-4} M), while 2×10^{-5} M ISN concentrations were ineffective. No difference between the individual isosorbide nitrates was noted. These results are in agreement with light transmission studies demonstrating that ISN are poor inhibitors of ARA-induced aggregation. Under ADP-induced aggregation TxB2 release occurred from endogenous arachidonate, but a ten-fold lower TxB2 release was noted when ADP instead of ARA was used as an aggregating agent. At high (5×10^{-4} M) and low (2×10^{-5} M) concentrations of ISDN, 2-ISMN and 5-ISMN thromboxane-release inhibiting strengths were 20, 68 and 55% and 0, 34 and 20%, respectively. Therefore, 2-ISMN is a more potent inhibitory agent of ADP-induced thromboxane release than 5-ISMN while ISDN is a poor inhibitor of arachidonate metabolism in ADP-stimulated platelets.

Discussion

The results of this study indicate that mononitrate metabolites of isosorbide dinitrate are inhibiting agents of both

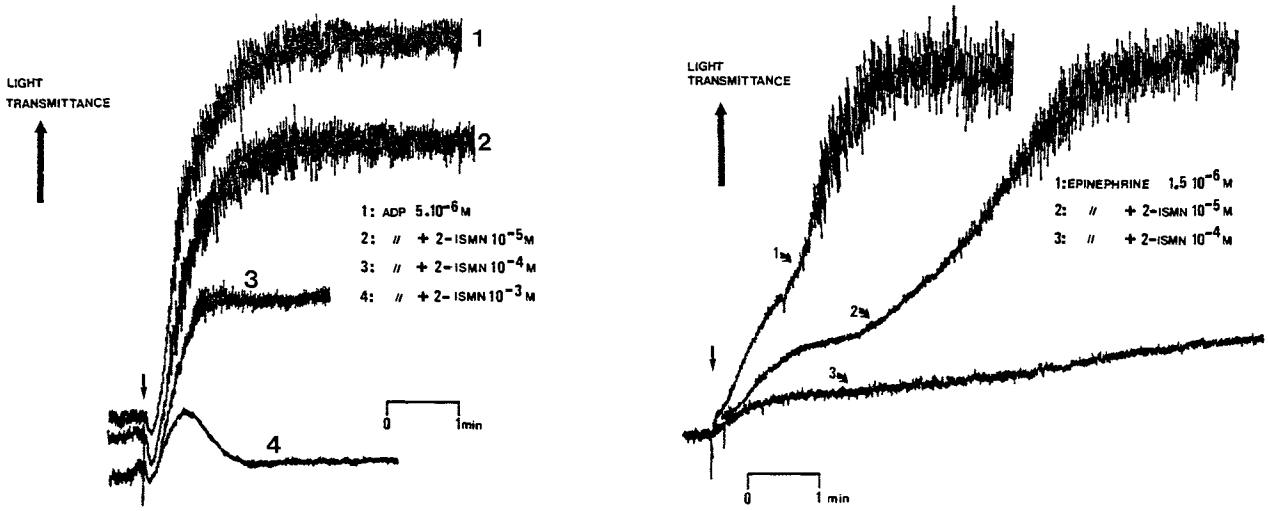

Fig. 1 Light transmission studies of 2-ISMN inhibitory effects on ADP- (left panel) and epinephrine- (right panel) induced platelet aggregation. The course of aggregation curves induced by 2-ISMN were recorded at various 2-ISMN concentration. 0.4 ml of PRP (3×10^5 platelets per microliter) was incubated with 0.05 ml of 2-ISMN at the defined concentration immediately prior the addition of 0.02 ml of ADP or epinephrine solution (arrow). Note that the isosorbide nitrates affected the extent of ADP-induced aggregation and the initial velocity of epinephrine-induced aggregation.

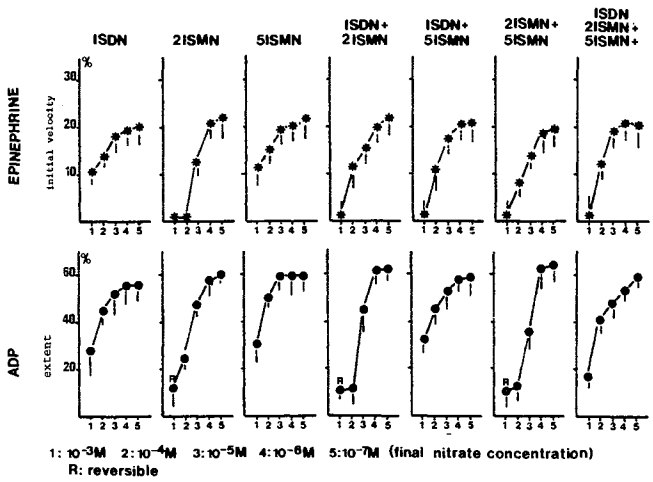

Fig. 2 Light transmission studies of ISN inhibitory effects against platelet aggregation induced by ADP (lower panel) and epinephrine (upper panel). Results are shown as percentage of the difference in light transmission between PRP and PPP, defining the extent of the aggregation reponse while the velocity was the percentage of the difference in LT between PRP and PPP, thirty seconds after the addition of the aggregating agent. The incubation procedure was as described in Fig. 1. ISN concentrations represent the sum of each nitrate added, when mixtures were used. The lowest ISN concentration served as control for EPI- and ADP-induced platelet aggregation.

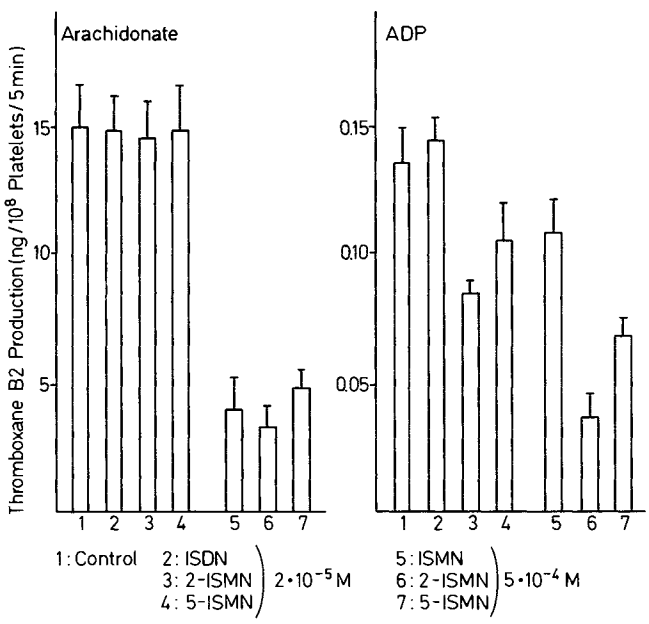

Fig. 3 Inhibition by ISN of platelet thromboxane B2 release during arachidonic acid (ARACHIDONATE) or ADP-induced aggregation. To 0.4 ml of washed platelet suspension (300×10^9 p/l) 0.05 ml of ISN was added prior to the addition of 0.05 ml arachidonate (5×10^{-5} M) or ADP (4.6×10^{-6} M). Incubation time was 5 min at 37°C.

ADP- and epinephrine-induced platelet aggregation. 2-ISMN appears to be more potent against platelet aggregation as compared to ISDN and 5-ISMN. In agreement with light transmission studies demonstrating that ISN are weak inhibitors of arachidonate-induced aggregation (9, 12), thromboxane B2 release from platelets was poorly affected by ISN.

Organic nitrates have their primary mode of action as direct inhibitors of vascular-type smooth muscle, but the mechanism of action of isosorbide nitrates and other nitrates is poorly understood. Therefore, the mechanisms sustaining most of the descriptive findings reported here remain to be elucidated. It has been hypothesized that ISDN-induced relaxation of coronary arterial smooth muscle is linked (causally or temporally?) to the formation of cGMP which in turn could induce phosphorylation of membrane proteins participating in calcium transport (19, 20). Contractile phenomena exist in both vascular smooth-muscle contraction and platelet aggregation, although the mechanism of granule content release and platelet shape changes are not well understood, but contractile processes are necessary events in the release reaction and further aggregation (21–24). With regard to ISN inhibition of contractile processes in vascular type smooth muscle, it is tempting to consider that ISN would interfere with the platelet contractile processes involved in platelet secretion and shape changes. However, the precise nature of ISN effects remains to be demonstrated, as well as where it takes place. Similarly, ISN anti-platelet activities through inhibition of contractile processes might stabilize platelet membranes. This would explain how ISN inhibits thromboxane release from endogenous arachidonate, e.g. from arachidonic acid esterified in phospholipids by preventing activation of phospholipases (25). Contrasting ISN inhibition of ADP-induced effect, the weak ISN inhibition of arachidonic acid-induced aggregation further supports the view that arachidonate, through its products, causes aggregation and fibrinogen binding to platelets by a mechanism independent of ADP, although ADP may enhance the reaction (25).

Our findings that ISN preferably affects the velocity more than the extent of epinephrine-induced aggregation suggest that ISN may alter the rate at which fibrinogen receptors are unmasked. In addition, the marked disparity between ISN effects against ADP- and EPI-induced aggregation supports the view that epinephrine-induced aggregation does not necessarily involve ADP-related processes (24, 26).

This study demonstrates that mononitrate metabolites of ISDN are active against platelet aggregation. Isosorbide dinitrate has a half-life in plasma of approximatively 30 min to 3 h, while mononitrate metabolites are eliminated from plasma less quickly, with half-lives from 3 to 6 h (27–30). It has been shown that plasma concentrations of ISDN, 2-ISMN and 5-ISMN increase gradually during chronic administration of the drug (27). In addition, ISDN accumulates in body tissue and is readily measurable for long periods after drug administration. At present, because of the widespread use of the long-acting or sustained-release forms of ISDN for chronic treatment, overall ISN concentrations as high as 1 to 4×10^{-6}M are reached in plasma. We report here that concentrations of 10^{-5}M total isosorbide nitrates were required to obtain inhibition of platelet activity. In addition to ISN promoting effects on prostacyclin synthesis (9, 12, 17) it is therefore apparent that ISDN plus its metabolites could account at least in part for the platelet inhibitory effects of ISDN administered *in vivo* (17). Yet, the biological and clinical relevance of such ISN effects on platelet activity as well as the concentrations required to observe it *in vitro*

remain to be determined. Aside from ISN cardiovascular effects, our results suggest that 2-ISMN is a potential anti-aggregating agent whose therapeutic use should be considered.

References

(1) Hillis, L. D., Braunwald, E. (1978) N. Engl. J. Med. 299, 695–702.
(2) Silver, M. D., Baroldi, G., Mariani, F. (1980) Circulation 61, 219–227.
(3) Dalen, J. E., Ockene, J. S., Alpert, J. S. (1982) Am. Heart J. 104, 1113–1124.
(4) Haerem, J. W. (1972) Atherosclerosis 15, 199–213.
(5) El-Maraghin, Genton, E. (1980) Circulation 62, 936–944.
(6) Distante, A., Maseri, A., Severi, S., Biagini, A., Chiercha, S. (1979) Am. J. Cardiol. 44, 533–540.
(7) Krantz, J. C. (1982) "Organic Nitrates". P. Needleman (Editor), Handbook of Experimental Pharmacology, Springer-Verlag, New York, 40, 5–7.
(8) Glusa, E., Markwarsr, F. (1974) Haemostasis 3, 249–256.
(9) Saxon, A., Kattlove, H. E. (1976) Blood 47, 957–961.
(10) Schafer, A. I., Alexander, R. W., Handin, R. I. Jr. (1980) Blood 55, 649–654.
(11) Rolland, P. H., Jouve, R., Pellegrin, E., Mercier, C., Serradimigni, A. (1984) Arteriosclerosis 4, 70–79.
(12) Hampton, J. R., Harrison, M. J. G., Honour, A. J., Mitchell, J. R. A. (1961) Cardiovasc. Res. 1, 101–107.
(13) Garratini, S. G. (1978) Raven Press, New York, 61–73.
(14) Levin, R. I., Jaffe, E. A., Weksler, B. B., Tack-Goldman, K. (1982) J. Clin. Invest. 67, 762–769.
(15) Rowan, R. M., Fraser, L., McDonald, G. A. (1979) Clin. Lab. Haemat. 1, 21–40.
(16) Rolland, P. H., Martin, P. M., Jacquemier, J., Rolland, A. M., Toga, M. (1980) J. Natl. Cancer Instit. 64, 1061–1070.
(17) Rolland, P. H., Bory, M., Leca, F., Sainsous, J., Gueydon, E., Juhan, I., Serradimigni, A., Cano, J. P. (1984) Prostagl. Leukotr. Med. 16, 333–346.
(18) Jouve, R., Rolland, P. H., Delboy, C., Mercier, C. (1984) Am. Heart J. 102, 45–52.
(19) Keith, R. A., Burkman, A. M., Sokoloski, T. D., Fertel, R. H. (1983) J. Pharmacol. Exp. Ther. 225, 29–34.
(20) Galvas, P. E., Disalvo, J. (1983) J. Pharmacol. Exp. Ther. 224, 373–378.
(21) Adelstein, R. S., Pollard, T. D. (1978) Prog. Hemostas. Thromb. 4, 37–58.
(22) Hathaway, D. R., Adelstein, R. S. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1653–1657.
(23) Carroll, R. C., Guerrard, J. M. (1982) Blood 59, 466–471.
(24) Verstraete, M. (1982) Haemostasis 12, 317–336.
(25) Harfenist, E. J., Guccione, M. A., Packham, M. A., Kingough-Rathbone, R. L., Mustard, J. F. (1982) Blood 59, 956–962.
(26) Peerschke, E. I. (1982) Blood 60, 71–77.
(27) Bruyneel, K., Rosseel, M. T., Bogaert, M. G. (1982) Arzneim. Forsch. Drug Res. 32, 769–773.
(28) Maddock, J., Lewis, P. A., Woodward, A., Massey, P. R., Kennedy, S. (1983) J. Chromatography 272, 129–136.
(29) Abshagen, U., Sporl-Radun, S. (1981) Europ. J. Clinical Pharmacol. 19, 424–429.
(30) Santoni, Y., Rolland, P. H., Cano, J. P. (1984) J. Chromatog. 306, 165–173.
(31) Reed, D. E., Akester, J. M., Prather, J. F., Tuckdsh, J. R., Maccurdy, D. H., Yeh, C. (1977) J. Pharmacol. Exp. Ther. 202, 32–37.

Evidence for an Acute Anti-magnesiuretic Effect of Triamterene Derivatives[3]

Helmut Priewer[1], Helga Kraft[1], and Ernst Mutschler[1,2]

Received: August 30, 1984; accepted: November 8, 1984.

Abstract: The acute natriuretic, antikaliuretic and antimagnesiuretic effects of two triamterene derivatives, carboxybutoxytriamterene ethyl amide and dimethylaminohydroxypropoxytriamterene (RPH 2823), are shown in male Wistar rats during urine collection periods of 1 to 2.5 h. In combination with furosemide both compounds reduce the potassium excretion that is caused by the loop diuretic. Furthermore, RPH 2823 strongly decreases the magnesiuresis after application of furosemide, and the ethyl amide derivative (25 μmol/kg) reduces the magnesium losses produced by 25 μmol/kg furosemide close to control values. The evaluation of dose-response curves gave further evidence for the hypothesis that the renal handling of K^+ and Mg^{2+} is coupled to some extent.

The potassium retaining diuretic triamterene is widely used in combination with more potent saluretics like thiazides or loop diuretics to prevent the potassium losses which are caused by these compounds. Besides producing pronounced kaliuresis, diuretic agents such as furosemide increase magnesium excretion (1, 2), which may be responsible for some of the side effects of loop diuretic therapy.

In addition to its antikaliuretic effect, triamterene possesses magnesium retaining properties in untreated and in saline-loaded rats (3, 4) and in normal subjects (5, 6). In contrast to its antikaliuretic effects, the antimagnesiuresis produced by triamterene is not observed immediately after dosing but appears several hours afterwards.

Synthesis and pharmacological testing of triamterene derivatives with electron rich substituents of the side chain revealed similar natriuretic effects and even increased antikaliuretic potencies compared with triamterene (7, 8). Screening experiments demonstrated an acute antimagnesiuretic effect of two of these compounds, carboxybutoxytriamterene ethyl amide and dimethylaminohydroxypropoxytriamterene (RPH 2823) (Fig. 1).

[1] Institute of Pharmacology, Department of Biochemistry, Pharmacy and Food Chemistry, University of Frankfurt, D-6000 Frankfurt, Fed. Rep. Germany.
[2] Correspondence to be addressed to Prof. Dr. Dr. E. Mutschler, Pharmakologisches Institut für Naturwissenschaftler, Theodor-Stern-Kai 7, Geb. 75A, 6000 Frankfurt, Fed. Rep. Germany.
[3] Part of the Ph. D. thesis of H. Priewer.